Bacterial lipopolysaccharides variably modulate *in vitro* biofilm formation of *Candida* species

H. M. H. N. Bandara,¹ O. L. T. Lam,² R. M. Watt,¹ L. J. Jin³
and L. P. Samaranayake¹

Correspondence

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

¹Oral Biosciences, Faculty of Dentistry, University of Hong Kong, 5/F Prince Philip Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong SAR

²Oral Rehabilitation, Faculty of Dentistry, University of Hong Kong, Sai Ying Pun, Hong Kong SAR

³Periodontology, Faculty of Dentistry, University of Hong Kong, Sai Ying Pun, Hong Kong SAR

The objective of this study was to evaluate the effect of the bacterial endotoxin LPS on *Candida* biofilm formation *in vitro*. The effect of the LPS of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Salmonella typhimurium* on six different species of *Candida*, comprising *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, was studied using a standard biofilm assay. The metabolic activity of *in vitro* *Candida* biofilms treated with LPS at 90 min, 24 h and 48 h was quantified by XTT reduction assay. Viable biofilm-forming cells were qualitatively analysed using confocal laser scanning microscopy (CLSM), while scanning electron microscopy (SEM) was employed to visualize the biofilm structure. Initially, adhesion of *C. albicans* was significantly stimulated by *Pseudomonas* and *Klebsiella* LPS. A significant inhibition of *Candida* adhesion was noted for the following combinations: *C. glabrata* with *Pseudomonas* LPS, *C. tropicalis* with *Serratia* LPS, and *C. glabrata*, *C. parapsilosis* or *C. dubliniensis* with *Salmonella* LPS ($P<0.05$). After 24 h of incubation, a significant stimulation of initial colonization was noted for the following combinations: *C. albicans*/*C. glabrata* with *Klebsiella* LPS, *C. glabrata*/*C. tropicalis*/*C. krusei* with *Salmonella* LPS. In contrast, a significant inhibition of biofilm formation was observed in *C. glabrata*/*C. dubliniensis*/*C. krusei* with *Pseudomonas* LPS, *C. krusei* with *Serratia* LPS, *C. dubliniensis* with *Klebsiella* LPS and *C. parapsilosis*/*C. dubliniensis* /*C. krusei* with *Salmonella* LPS ($P<0.05$). On further incubation for 48 h, a significant enhancement of biofilm maturation was noted for the following combinations: *C. glabrata*/*C. tropicalis* with *Serratia* LPS, *C. dubliniensis* with *Klebsiella* LPS and *C. glabrata* with *Salmonella* LPS, and a significant retardation was noted for *C. parapsilosis*/*C. dubliniensis*/*C. krusei* with *Pseudomonas* LPS, *C. tropicalis* with *Serratia* LPS, *C. glabrata*/*C. parapsilosis*/*C. dubliniensis* with *Klebsiella* LPS and *C. dubliniensis* with *Salmonella* LPS ($P<0.05$). These findings were confirmed by SEM and CLSM analyses. In general, the inhibition of the biofilm development of LPS-treated *Candida* spp. was accompanied by a scanty architecture with a reduced numbers of cells compared with the profuse and densely colonized control biofilms. These data are indicative that bacterial LPSs modulate *in vitro* *Candida* biofilm formation in a species-specific and time-dependent manner. The clinical and the biological relevance of these findings have yet to be explored.

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INTRODUCTION

Most micro-organisms exist in nature as complex communities attached to surfaces rather than as isolated free-floating

planktonic organisms. These structurally and functionally complex communities, termed biofilms, consist of one or more microbial species encased in an extracellular polymeric substance, and attached to one another or to a solid surface (Samaranayake & MacFarlane, 1990). Biofilms, in comparison to their planktonic counterparts, are resistant to antimicrobials (Mah & O'Toole, 2001; O'Toole *et al.*, 2000), and are thought to be involved in up to 65 % of all human microbial infections (McLean *et al.*, 2004).

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

Tables of XTT reduction analysis data are available as supplementary material with the online version of this paper.

Biofilms quite frequently comprise multiple species of either bacteria alone (monospecies) or bacteria together with fungi such as *Candida* (multispecies) (Bandara *et al.*, 2009; Nobile & Mitchell, 2007). The dimorphic fungus *Candida* resides in human oral cavities, the vagina, the gastrointestinal tract, on skin and on other mucosal surfaces as a commensal organism (Jenkinson & Douglas, 2002; Samaranayake & MacFarlane, 1990). Though the genus *Candida* comprises more than 150 yeast species, only a few, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida kefyr*, *Candida glabrata* and *Candida guilliermondii*, are recognized as medically important pathogens (Odds, 1988). *C. albicans* is the fourth leading cause of nosocomial bloodstream infection in patients in medical intensive care units (Pittet & Wenzel, 1995; Richards *et al.*, 1999), and causes superficial to deep infections in neonates, diabetics and immunosuppressed patients (Eubanks *et al.*, 1993; Pfaller, 1996).

LPS, a bacterial endotoxin, is an amphiphilic molecule, which is heterogeneous both in size and composition (Aurell & Wistrom, 1998). It is an outer cell wall component of Gram-negative bacteria that initiates septic shock in humans and animals through its proinflammatory and immunomodulatory properties (Aurell & Wistrom, 1998; Morrison & Ryan, 1987; Rietschel *et al.*, 1982). LPS also induces a variety of responses in monocytes and polymorphonuclear cells, which may play a role in the mechanism of endotoxic shock (Michalek *et al.*, 1980; Tracey *et al.*, 1987); other manifestations of LPS may play a role in the pathophysiology of infections (Morrison & Ryan, 1987; Young, 1985).

The interactions between LPS and *Candida* have been sparsely investigated. In previous studies, Henry-Stanley *et al.* (2003) demonstrated that *Escherichia coli* LPS enhances the lethality of parenterally administered *C. albicans*. Palma *et al.* (1992), in an *in vitro* study, showed that LPS from *E. coli*, *Serratia marcescens* and *Salmonella typhimurium*, at doses ranging from 1 to 100 ng ml⁻¹, strongly enhanced the inhibitory response of human polymorphonuclear leukocytes against the growth of *C. albicans*. Furthermore, the number of yeasts ingested per neutrophil, as well as the number of neutrophils capable of ingesting fungal cells, was markedly increased as a result of LPS exposure (Palma *et al.*, 1992). Our group recently demonstrated, for the first time, the direct modulatory effect of *E. coli* LPS on different stages of *Candida* biofilm formation. *E. coli* LPS significantly suppressed the initial colonization of *Candida dubliniensis*, while it enhanced the initial colonization of *C. tropicalis* and maturation of *C. parapsilosis* biofilms (Bandara *et al.*, 2009).

Numerous *in vivo* and *in vitro* studies have shown that various bacteria and *Candida* have complex interactions in mixed-species biofilms. This suggests that bacterial infections may play a modulatory role in *Candida* infections (Bandara *et al.*, 2009; Hedderwick *et al.*, 2000; Peres-Bota *et al.*, 2004). However, the role of the major Gram-negative

bacterial endotoxin, LPS, in mixed infections is yet to be fully unravelled. Though a few studies have been conducted, none have been designed to illustrate the effect of LPSs on the process of biofilm formation of this yeast. Furthermore, the role of different Gram-negative bacterial LPS on candidal biofilms has not been evaluated. Additionally, the majority of the previous studies have focused only on *C. albicans* and, to the best of our knowledge, there are no studies pertaining to non-*albicans* *Candida* spp. biofilms.

Hence, the aims of this study were to evaluate the effect of LPS derived from *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Salmonella typhimurium* on six different *Candida* species, including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis* and *C. krusei*, in different stages of biofilm formation, by both quantitative assays (XTT reduction assay), and qualitative evaluations using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

METHODS

Experimental design. The study comprised a series of multiple experiments to evaluate the effect of LPS derived from different Gram-negative bacteria as described below, on *Candida* spp. biofilm formation, quantitatively with XTT reduction assay, and qualitatively with CLSM and SEM, at three different time intervals, 90 min, 24 h and 48 h. These time intervals corresponded to the adhesion phase, initial colonization phase and the maturation phase of *Candida* biofilms, respectively. These latter definitions and terminology are used consistently in the following sections of the paper.

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

LPS. LPSs purified by phenol extraction from *K. pneumoniae* ATCC 15380 (catalogue no. L4268), *Serratia marcescens* ATCC 21639 (catalogue no. L6136), *Salmonella typhimurium* ATCC 7823 (catalogue no. L6511), and *P. aeruginosa* ATCC 27316 (catalogue no. L9143) were purchased in the form of lyophilized powder from Sigma Aldrich and stored 2–8 °C until use.

Growth media. Sabouraud dextrose agar and yeast nitrogen base (YNB) solution supplemented with 100 mM glucose were used for culturing *Candida* species.

Microbial inocula. Prior to each experiment, *Candida* spp. were subcultured on Sabouraud dextrose agar for 18 h at 37 °C. A loopful of this overnight *Candida* growth was inoculated into YNB 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 (pH 7.2, Ca²⁺ and Mg²⁺ free; Sigma) and resuspended. PBS without calcium and magnesium ions was used to wash and rinse suspended cells as these ions facilitate cell binding and clumping, which will mask the effect of LPS on *Candida* during biofilm development. Concentrations of *Candida* spp. were adjusted 1 × 10⁷ cells ml⁻¹ by spectrophotometry and confirmed by haemocytometric counting.

Biofilm formation. *Candida* biofilms were prepared as described by Jin *et al.* (2004), with some modifications. Commercially available pre-sterilized, polystyrene, flat bottom 96-well microtitre plates (Iwaki) were used. At first, 100 μ l standard cell suspension of *Candida* spp. (10^7 organisms ml^{-1}) was prepared and transferred into the wells of a microtitre plate, and the plate 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. After the initial adhesion phase, the cell suspensions were aspirated and each well was washed twice with PBS to remove loosely adherent cells. A total of 200 μ l YNB was transferred to each well and the plate reincubated for 24 and 48 h, and the wells washed twice and three times at the respective time intervals with PBS to eliminate traces of YNB. Thus, the effect of bacterial LPS on *Candida* was studied at 90 min, 24 h and 48 h time intervals.

Quantitative analyses – evaluation of LPS impact using XTT reduction assay. Freshly obtained LPS (1 mg ml^{-1} ; Sigma Aldrich) and YNB supplemented with 100 mM glucose were prepared to yield a final dilution of LPS ranging from 100 $\mu\text{g ml}^{-1}$ to 1 ng ml^{-1} . LPS was replaced with sterile PBS in the control samples. *Candida* biofilms were formed in three different 96-well plates to evaluate the effect of LPS at 90 min, 24 h and 48 h (Fig. 1) as described below.

First, a *Candida* suspension (100 μ l each) resuspended in test and control media (see above) was 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 μ l test and control media were 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 further 24 h for the maturation phase study.

At the end of incubation, the supernatant was removed and wells washed twice with sterile PBS to remove loosely adherent cells. A standard XTT reduction assay was performed thereafter as described by Jin *et al.* (2004) to measure the metabolic activity of the biofilms. In brief, commercially available XTT powder (Sigma) was dissolved in PBS at a final concentration of 1 mg ml^{-1} , then 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 assay. XTT solution was thawed and mixed with menadione solution at 20:1 (v/v) immediately before the assay. Thereafter, 158 μ l PBS, 40 μ l XTT and 2 μ l menadione solution were added into each well containing prewashed biofilms and incubated in the dark for 3 h at 37 °C. The colour change was measured by absorbance with a microtitre plate reader (Spectra Max 340 tunable microplate reader; Molecular devices) at 492 nm. All assays were carried out in triplicate on three different occasions.

Qualitative analyses. CLSM (Jin *et al.*, 2005) and SEM were used to observe the ultrastructure of test and control *Candida* biofilms (Fig. 1).

CLSM. Commercially available presterilized flat-bottomed six-well plates (Iwaki) and presterilized plastic coverslips (Thermanox; Nalge Nunc International) (Ramage *et al.*, 2001) were used to prepare biofilms as described above. Presterilized coverslips were placed in the wells of a six-well plate, *Candida* suspensions added and the plate incubated for 90 min (the adhesion phase) in an orbital shaker (75 r.p.m.) at 37 °C. Thereafter, the supernatant was removed, washed twice with PBS, fresh YNB added and incubated for 24 h (initial colonization) or 48 h (maturation) under the same environmental conditions. At the end of each time interval, the prewashed coverslips were stained with LIVE/DEAD stain (LIVE/DEAD BacLight Bacterial viability kit; Invitrogen) (Jin *et al.*, 2005). The biofilm was then analysed by fluorescent microscopy (using a confocal laser scanning microscope).

SEM. For SEM, we prepared *Candida* species biofilms (both control and LPS treated) on custom-made, tissue culture-treated, polystyrene coverslips (prepared in six-well plates) 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. Then the 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 with a scanning electron microscope (Philip XL30CP) in high-vacuum mode at 10 kV, and the images processed.

Statistical analysis. Statistical analysis was performed using SPSS software (version 16.0). The Mann–Whitney U-test was performed to compare significant differences between the control and each test sample of the candidal biofilm. Data from all *Candida* spp. at different time points (90 min, 24 h and 48 h) were pooled and evaluated against a concentration gradient of different LPS using 2-sample *t*-test. A similar statistical test was used to analyse pooled data from all LPS experiments at a concentration gradient against different *Candida* spp. at different time points (90 min, 24 h and 48 h). A *P* value of <0.05 was considered statistically significant.

RESULTS

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

In the early biofilm development phase, after 90 min of incubation, some of the LPS-treated *Candida* species demonstrated significant changes in metabolic activity

Fig. 1. Summary of the experimental design. *Candida* biofilm formation was evaluated at three different time intervals: 0–90 min (adhesion phase), 90 min–24 h (initial colonization phase) and 24–48 h (maturation phase). At the commencement of each time interval, varying concentrations of LPS were added to *Candida* biofilms in 96-well plates; at the end of each phase, quantitative and qualitative analyses were performed using the XTT reduction assay, and CLSM and SEM, respectively.

compared with their controls. With *Pseudomonas* LPS, *C. albicans* showed significant enhancement of its metabolic activity at $10\ \mu\text{g ml}^{-1}$ and in contrast, *C. glabrata* was significantly inhibited at $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$, $100\ \text{ng ml}^{-1}$ and $10\ \text{ng ml}^{-1}$. Adhesion of the other four *Candida* species were unaffected with *Pseudomonas* LPS treatment. With *Serratia* LPS, *C. tropicalis* was the only species affected out of all six *Candida* species, and demonstrated significantly reduced XTT readings at the concentrations of $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$ and $1\ \mu\text{g ml}^{-1}$. With *Salmonella* LPS, a similar effect was observed in the adhesion phase of *C. glabrata* at $100\ \mu\text{g ml}^{-1}$ and $10\ \mu\text{g ml}^{-1}$, *C. parapsilosis* at $100\ \mu\text{g ml}^{-1}$ and *C. dubliniensis* at $10\ \mu\text{g ml}^{-1}$, while the adhesion of *C. albicans*, *C. tropicalis* and *C. krusei* remained unaffected. In contrast, *Klebsiella* LPS significantly increased the metabolic activity of only *C. albicans* early biofilms at $10\ \mu\text{g ml}^{-1}$ concentration (Table 1, Supplementary Tables S1, S2, S3, S4, S5 and S6 available with the online journal).

However, after prolonged incubation, for 24 h, beyond the initial colonization phase of the biofilms, significant variations in XTT readings were seen in all *Candida* spp. Thus, upon *Pseudomonas* LPS treatment, *C. glabrata* at $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$ and $1\ \text{ng ml}^{-1}$, and *C. dubliniensis* and *C. krusei* at $100\ \mu\text{g ml}^{-1}$ showed significantly reduced XTT values. *C. albicans*, *C. tropicalis* and *C. parapsilosis* showed uninterrupted colonization at this stage. A stimulatory effect was detected in *C. krusei* biofilms treated with $10\ \mu\text{g Serratia}$ LPS ml^{-1} and the colonization of remaining five *Candida* species was unaffected. *C. dubliniensis* treated with $100\ \mu\text{g Klebsiella}$ LPS ml^{-1} also demonstrated a significant reduction in XTT readings. In contrast, *Klebsiella* LPS-treated biofilms of *C. albicans* at $100\ \mu\text{g ml}^{-1}$, and *C. glabrata* at $10\ \mu\text{g ml}^{-1}$ and $1\ \mu\text{g ml}^{-1}$, showed a significant stimulation of metabolic activity, whilst *C. tropicalis*, *C. parapsilosis* and *C. krusei* showed normal biofilm growth. *Salmonella* LPS also had a similar stimulatory effect on *C. glabrata* at $100\ \text{ng ml}^{-1}$, on *C. tropicalis* at $100\ \mu\text{g ml}^{-1}$ and $10\ \text{ng ml}^{-1}$, and on *C. krusei* at $1\ \text{ng ml}^{-1}$. However, *Salmonella* LPS significantly lowered the metabolic activity of *C. parapsilosis* at $100\ \text{ng ml}^{-1}$, and *C. dubliniensis* and *C. krusei* at $100\ \mu\text{g ml}^{-1}$. *C. albicans* could withstand the effects of *Salmonella* LPS at this stage (Table 1).

On further incubation for 48 h, *Pseudomonas* LPS demonstrated a significant suppression of metabolic activities of *C. parapsilosis* (at $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$ and $100\ \text{ng ml}^{-1}$), *C. dubliniensis* (at $100\ \mu\text{g ml}^{-1}$) and *C. krusei* (at $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$, $100\ \text{ng ml}^{-1}$ and $1\ \text{ng ml}^{-1}$); although, the maturation of *C. albicans*, *C. glabrata* and *C. tropicalis* biofilms were unaffected in the presence of *Pseudomonas* LPS. In contrast, when treated with *Serratia* LPS, an elevated metabolic activity could be observed in *C. glabrata* (at $100\ \mu\text{g ml}^{-1}$ and $10\ \text{ng ml}^{-1}$), and *C. tropicalis* (at $100\ \mu\text{g ml}^{-1}$, $10\ \mu\text{g ml}^{-1}$ and $1\ \mu\text{g ml}^{-1}$). Conversely, *C. parapsilosis* showed the opposite response at $100\ \mu\text{g ml}^{-1}$ though *C. albicans*, *C. dubliniensis* and *C. krusei* biofilms were unaffected. When treated with *Klebsiella* LPS, *C. glabrata* (at $10\ \mu\text{g ml}^{-1}$), and *C. dubliniensis* (at $100\ \mu\text{g ml}^{-1}$) demonstrated significantly

low XTT readings compared to the controls, though a significantly higher reading was noted with *C. parapsilosis* (at $100\ \mu\text{g ml}^{-1}$ and $10\ \mu\text{g ml}^{-1}$) and *C. dubliniensis* (at $100\ \text{ng ml}^{-1}$ and $10\ \text{ng ml}^{-1}$). The remaining three *Candida* species tested were unaffected. A significant increase in the metabolic activity of *C. glabrata* was observed after treatment with *Salmonella* LPS (at $100\ \mu\text{g ml}^{-1}$, $1\ \mu\text{g ml}^{-1}$ and $100\ \text{ng ml}^{-1}$ concentrations). In contrast, a lower XTT reading was detected in *C. dubliniensis* biofilms treated with $100\ \mu\text{g Salmonella}$ LPS ml^{-1} . *C. albicans*, *C. parapsilosis* and *C. dubliniensis* biofilms showed normal maturation despite the treatment with *Salmonella* LPS (Table 1).

We further analysed the pooled data from the foregoing experiments due to the high variability of the results. This was performed in two ways: first, the data from all *Candida* species against LPS of different Gram-negative bacterial species were pooled and analysed; second, the data from all bacterial LPS against six different *Candida* spp. were pooled and analysed.

In the first analysis, after 90 min of incubation (adhesion phase) *Salmonella* LPS at $100\ \mu\text{g ml}^{-1}$ and $10\ \mu\text{g ml}^{-1}$ significantly suppressed the metabolic activity of *Candida* spp. in general. However, the remaining types of LPS tested, at any given concentration, had no significant effects on the adhesion (90 min), initial colonization (24 h) or biofilm maturation (48 h) of *Candida* species.

Furthermore, when the data from all the tests of bacterial LPS against six different *Candida* spp. were pooled and analysed, we noted that the metabolic activity of the *C. albicans* 24 h biofilm was significantly higher when treated with $100\ \mu\text{g LPS ml}^{-1}$. In contrast, at a similar concentration of LPS, *C. dubliniensis* 24 h and 48 h biofilms, and *C. krusei* 24 h biofilm demonstrated a significant reduction in the metabolic activity. *C. glabrata*, *C. tropicalis* and *C. parapsilosis* in all three stages of biofilm formation could withstand any given concentration of LPS without any significant effect.

CLSM

CLSM with LIVE/DEAD stain showed an increase in *C. albicans* blastospores after incubation with *Pseudomonas* LPS for 90 min, confirming the data from XTT reduction assay (Fig. 2a, b). Furthermore, LPS-treated *C. albicans* were uniform in size, clumped and showed a higher proportion of budding yeasts compared to its control (Fig. 2b), whilst a considerable number of dead yeasts were visible among scattered *C. albicans* in the control (Fig. 2a).

After 24 h of incubation (initial colonization phase), growth suppression of *C. krusei* by *Salmonella* LPS was clearly seen by CLSM with LIVE/DEAD stain, confirming the XTT reduction assay data (Fig. 2c, d). Thus a few, scant *C. krusei* blastospores were scattered in the microscopic field. Yeasts were small in size and few showed budding morphology. Some dead and degrading cells were also present in sparsely visible matrix (Fig. 2d). In contrast, its

Table 1. Percentage modulation of biofilm growth of six different *Candida* spp. by six different concentrations of LPS from four different bacteria at varying time intervals

Biofilm activity was determined by XTT reduction assay at 90 min, 24 h and 48 h biofilm formation. The Mann–Whitney U test was performed and a *P* value <0.05 was considered as statistically significant. Positive percentages indicate LPS concentrations showing significant biofilm stimulation. Negative percentages indicate LPS concentrations showing a significant suppression. Due to the complexity and multitude of data, only the combinations that showed significant findings are indicated in the table.

<i>Candida</i> spp.	Time	<i>Pseudomonas</i> LPS						<i>Serratia</i> LPS						<i>Klebsiella</i> LPS						<i>Salmonella</i> LPS					
		100 µg ml ⁻¹	10 µg ml ⁻¹	1 µg ml ⁻¹	100 ng ml ⁻¹	10 ng ml ⁻¹	1 ng ml ⁻¹	100 µg ml ⁻¹	10 µg ml ⁻¹	1 µg ml ⁻¹	100 ng ml ⁻¹	10 ng ml ⁻¹	1 ng ml ⁻¹	100 µg ml ⁻¹	10 µg ml ⁻¹	1 µg ml ⁻¹	100 ng ml ⁻¹	10 ng ml ⁻¹	1 ng ml ⁻¹	100 µg ml ⁻¹	10 µg ml ⁻¹	1 µg ml ⁻¹	100 ng ml ⁻¹	10 ng ml ⁻¹	1 ng ml ⁻¹
<i>C. albicans</i>	90 min		+4												+13										
	24 h													+18											
	48 h																								
<i>C. glabrata</i>	90 min	-10	-7		-8	-8															-64	-57			
	24 h	-15	-6				-12								+7	+7							+3		
	48 h							+6				+10			-15						+9		+7	+7	
<i>C. tropicalis</i>	90 min							-15	-19	-13															
	24 h																							+13	
	48 h							+30	+18	+12															
<i>C. parapsilosis</i>	90 min																				-18				
	24 h																							-10	
	48 h	-23	-13		-8			-12						+45	+71										
<i>C. dubliniensis</i>	90 min																					-4			
	24 h													-25							-29				
	48 h													-24			+12	+9			-15				
<i>C. krusei</i>	90 min																								
	24 h	-15							+11												-40				+13
	48 h	-19	-12		-13		-11

Fig. 2. CLSM images of *Candida* spp. biofilms treated with Gram-negative bacterial LPS (magnification $\times 40$) (stained using a LIVE/DEAD BacLight bacterial viability kit; Invitrogen). Live cells are stained in green and dead cells in red. (a, b) Adhesion of *C. albicans* at 90 min: (a) control, (b) treated with *Pseudomonas* LPS ($10 \mu\text{g ml}^{-1}$). Note the enhancement of *C. albicans* adhesion due to *Pseudomonas* LPS in comparison to its untreated counterpart. (c, d) Initial colonization of *C. krusei* at 24 h: (c) control, (d) treated with *Salmonella* LPS ($100 \mu\text{g ml}^{-1}$). Note the profuse, healthy control biofilm of *C. krusei*, compared with the *Salmonella* LPS-treated biofilm with scanty growth. (e, f) Initial colonization of *C. glabrata* at 24 h: (e) control, (f) treated with *Pseudomonas* LPS ($100 \mu\text{g ml}^{-1}$). Note the suppression of *C. glabrata* growth in the *Pseudomonas* LPS-treated sample. (g, h) Maturation of *C. dubliniensis* at 48 h: (g) control; (h) treated with *Salmonella* LPS ($100 \mu\text{g ml}^{-1}$). Note the suppression of *C. dubliniensis* by *Salmonella* LPS.

control counterpart showed a higher proportion of uniform and elongated *C. krusei* blastospores arranged in clusters (Fig. 2c). Similarly, at the same time point (Fig. 2e, f), groups of irregular shaped blastospores of *C. glabrata* were visible in the *Pseudomonas* LPS-treated ($100\ \mu\text{g ml}^{-1}$) sample (Fig. 2f), while the *C. glabrata* control was densely populated with uniform yeasts (Fig. 2e).

After 48 h of incubation (maturation phase), sparsely distributed *C. dubliniensis* blastospores were noted in biofilms treated with *Salmonella* LPS, reconfirming the foregoing quantitative (XTT) findings (Fig. 2g, h). Yeasts were anisotropic, with some cellular debris in the background (Fig. 2h) compared to the control where high numbers of *C. dubliniensis* yeast forms and some hyphae were evident (Fig. 2g).

SEM

After 90 min of incubation (adhesion phase), increased numbers of blastospores adherent to the polystyrene surface were noted in *C. albicans* treated with *Pseudomonas* LPS ($100\ \mu\text{g ml}^{-1}$) (Fig. 3a, b). Yeasts of varying sizes were noted and some with irregular cell profiles appeared to be degrading (Fig. 3b). In contrast, the *C. albicans* control had lesser numbers of yeasts of more uniform size, and healthier than those treated with LPS (Fig. 3a).

After 24 h of incubation, the *C. dubliniensis* control characteristically exhibited a profuse, dense growth; although, the extracellular matrix was scarcely visible under SEM. Yeasts were uniform in appearance (Fig. 3c). However, *C. dubliniensis* treated with *Pseudomonas* LPS ($100\ \mu\text{g ml}^{-1}$) showed a scanty biofilm with reduced numbers of cell layers and reduced cell regularity. Yeasts were different in size and shape (Fig. 3d).

After 48 h of incubation for biofilm maturation, *C. krusei* biofilm controls demonstrated elongated, more uniform yeasts with a substantial amount of extracellular matrix. Some dead and degrading cells could also be observed under SEM (Fig. 3e). In contrast, *C. krusei* treated with *Pseudomonas* LPS ($100\ \mu\text{g ml}^{-1}$) showed a scanty biofilm with elongated blastospores and sparse extracellular matrix (Fig. 3f).

DISCUSSION

Candida is a polymorphic fungus that frequently causes systemic infections in critically ill patients (Rangel-Frausto *et al.*, 1999). Despite advances in antifungal therapy, the upsurge of the incidence of fungaemia in such communities has been noted with concern (Charles *et al.*, 2003; Pappas *et al.*, 2003; Peres-Bota *et al.*, 2004). This ubiquitous yeast is also the fourth most common cause of nosocomial blood-stream infection (Pappas *et al.*, 2003; Rangel-Frausto *et al.*, 1999), and the agent of a significant proportion of crude (61%) and attributable (49%)

mortality in critically ill patients in North America (Charles *et al.*, 2003).

It was recently estimated that Gram-negative bacteria are involved in half of the cases of sepsis (approx. 500 000 cases of sepsis) that occur annually in the USA. Up to 60% of these cases tend to end in septic shock (Rangel-Frausto, 1999). Gram-negative bacterial LPS, a potent microbial toxin, and its proinflammatory properties are well known to play a critical role in the pathogenesis of such septic shock (Luchi & Morrison, 2000).

Though *C. albicans* is the most commonly encountered organism from the genus *Candida* in clinical specimens, the incidence of non-*albicans Candida* is rising (Pappas *et al.*, 2003; Peres-Bota *et al.*, 2004; Rangel-Frausto *et al.*, 1999; Snyderman, 2003; Wisplinghoff *et al.*, 2004). Furthermore, there is evidence to indicate that bacterial co-infection or superinfection play a critical role in *Candida* infections (Bandara *et al.*, 2009; Peres-Bota *et al.*, 2004). Some have also reported that Gram-negative bacterial LPS has direct (Bandara *et al.*, 2009) and indirect effects (Henry-Stanley *et al.*, 2003) on *Candida* biofilm formation, and on candidal infections.

We therefore evaluated the quantitative and qualitative effect of four different Gram-negative bacterial LPSs from *P. aeruginosa*, *Serratia marcescens*, *K. pneumoniae* and *Salmonella typhimurium* on various stages of biofilm development in six different human pathogenic *Candida* species. Our results are indicative that different LPSs modulate the various stages of biofilm development of specific *Candida* spp. to varying degrees.

Using the XTT reduction assay, we report here for what is believed to be the first time, the quantitative effects of four different bacterial LPS on six different *Candida* species at three different time points in biofilm development. When taken individually, each type of LPS tested here had significant effects, either inhibitory or stimulatory, on *Candida* species. In general, the effect elicited by LPS was time and concentration dependent. *Salmonella* and *Pseudomonas* LPS had the strongest overall effect on *Candida*, while *Serratia* LPS had the least. The effect of *Pseudomonas* LPS was mainly inhibitory while, the contrary was true for *Salmonella* LPS. When the phase of biofilm development was evaluated, *Salmonella* LPS had the strongest effect on *Candida* adhesion, being inhibitory in nature and the least effect was noted with *Klebsiella* LPS. *Salmonella* LPS effect was mixed, with the strongest effect on the initial candidal colonization (24 h) stage. *Serratia* LPS showed the weakest effect at this stage by only inhibiting *C. krusei*.

Pseudomonas LPS had the strongest suppressive effect on the maturation phase *Candida* biofilms. In contrast, *Serratia* and *Salmonella* LPS showed the highest, stimulatory, effect on the latter phase of *Candida* biofilms. Despite these specific variations, *Salmonella* LPS significantly inhibited the adhesion of *Candida* spp. at 100 and $10\ \mu\text{g ml}^{-1}$.

Fig. 3. SEM images of *Candida* spp. biofilms – control biofilms and biofilms treated with Gram-negative bacterial LPSs (magnification $\times 2000$). (a, b) Adhesion of *C. albicans* (90 min): (a) control, (b) treated with *Pseudomonas* LPS ($10 \mu\text{g ml}^{-1}$). Note the LPS-treated *C. albicans* cells are heterogeneous in size and more populated in comparison to the control. (c, d) Initial colonization of *C. dubliniensis* (24 h): (c) control, (d) treated with *Pseudomonas* LPS ($100 \mu\text{g ml}^{-1}$). Note the LPS-treated *C. dubliniensis* blastospores are less in number, different in shape and scantily distributed in comparison to the more uniform and densely colonized control. (e, f) Maturation of *C. krusei* (48 h): (e) control, (f) treated with *Pseudomonas* LPS ($100 \mu\text{g ml}^{-1}$). Note the LPS-treated *C. krusei* biofilm is less organized with scattered cells compared to the control.

In general, when the species-specific variations of *Candida* in response to LPS were considered, *C. glabrata* was the most susceptible to the LPS effect at any time point of biofilm development; *C. albicans* and *C. tropicalis* were the most resistant. At 90 min, the adhesion of *C. glabrata* was essentially inhibited by LPS. The effect was mixed at 24 h, while it was stimulatory at 48 h. Despite such species-specific findings, we noted that the initial colonization of *C. albicans* was significantly enhanced by $100 \mu\text{g LPS ml}^{-1}$.

Our results substantiate the concept that Gram-negative bacterial LPSs in general have a direct modulatory effect on *Candida* biofilm development. The current data also validate the results of our previous study, which showed that *E. coli* LPS has a modulatory effect on *Candida* biofilm formation (Bandara *et al.*, 2009). In contrast, Palma *et al.* (1992) have reported that LPSs of *E. coli*, *Serratia marcescens* and *Salmonella typhimurium* had no direct effect on *Candida* growth at any concentration.

The phenotypic variations of LPS could be one important factor that affects its species-specific functional diversity. There is evidence, including the current observations, to support the concept that LPS diversity affects its biological activity. For instance, LPS activity can vary substantially depending upon both the type of the organism and the method of its extraction (Morrison & Leive, 1975). The presence of outer-membrane proteins in association with LPS (Morrison *et al.*, 1976), the specific subunit composition of LPS (Vukajlovich & Morrison, 1985) and the presence of O-antigen carbohydrates that are linked to the LPS (Morrison *et al.*, 1987) can all determine the quality of this lipid-carbohydrate complex. Sabra *et al.* (2003) explained that the phenotypic variations in the relative expression of two chemically distinct types of LPS of *P. aeruginosa* could be an important mechanism that alters surface characteristics in mediating adhesion and survival under extreme conditions (Makin & Beveridge, 1996; Sabra *et al.*, 2003). It should also be noted that, apart from such chemical properties, the method of LPS preparation may have an effect on its bioactivity and toxicity (Luchi & Morrison, 2000; Morrison & Leive, 1975).

Although limited data are available on the effect of LPS *in vitro*, there are a number of *in vivo* studies demonstrating the variable effects of LPS on *Candida*. Burd *et al.* (1992) noted that when mice were injected with *C. albicans* along with viable *E. coli* or *E. coli* LPS intraperitoneally, both *E. coli* as well as LPS enhanced the virulence of *C. albicans*. In a similar study, Klaerner *et al.* (1997) also noted that mice injected with *E. coli* or LPS had increased number of *C. albicans* in kidney tissue suggesting the promotive effect of LPS on *C. albicans* (Akagawa *et al.*, 1995). As in the current study, species specificity of LPS appears to influence its outcome in the host. On the contrary, Hess *et al.* (2009) noted that the tissue burden of *C. glabrata* in mice was decreased in the presence of *E. coli* and its LPS. Henry-Stanley *et al.* (2003) demonstrated that LPS may protect against extraintestinal dissemination of orally inoculated *C. albicans* by inhibiting the adhesion of yeasts to intestinal epithelium.

It should be borne in mind that proinflammatory and immunomodulatory properties of LPS *in vivo* are major factors determining its indirect effect on *Candida* (Morrison & Ryan, 1987; Rietschel *et al.*, 1982). Palma *et al.* (1992) noted in an *in vitro* study that LPS from *E. coli*, *Serratia marcescens* and *Salmonella typhimurium*, at doses from 1 to 100 ng ml⁻¹, strongly enhanced the growth inhibition of *C. albicans* caused by human polymorphonuclear leukocytes.

Ultrastructural views of both LPS-treated and untreated *Candida* biofilms further confirmed the results of our XTT reduction assays. Generally, when a specific stage of *Candida* biofilm formation was suppressed by LPS, the biofilm tended to display a rather scanty architecture with a low proportion of budding yeasts, and a preponderance of dead and degrading cells. *Candida* blastospores were small

in size and heterogeneous in shape in comparison to the more uniform, confluent, and relatively well-defined control biofilms. On the contrary, when a specific stage of *Candida* biofilm formation was stimulated by LPS, the biofilm was richly populated by more isoform blastospores and a large proportion of live and budding *Candida*, while the controls were devoid of such features.

In clinical terms, our data are indicative that individuals with systemic candidiasis superinfected with Gram-negative bacteria need prompt treatment to eradicate both types of organisms and to neutralize the action of LPS (Akagawa *et al.*, 1995). If indeed some form of LPS does modify the pathogenicity and virulence of *Candida*, as observed in our study, then it is tempting to suggest that a modified non-toxic form of LPS would be an alternative treatment option for reduction of the incidence of systemic candidiasis in high-risk patients (Henry-Stanley *et al.*, 2003).

In summary, our studies, focused mainly on the effect of various Gram-negative bacterial LPSs on different stages of *Candida* spp. biofilm development, are indicative that LPS has significant and direct modulatory effects on various stages of *Candida* biofilm development in a species-specific manner. It is also clear that some *Candida* species are able to withstand the effect of LPS more than others as the effect of LPS on *Candida* was specific and diverse. Further work is necessary to explain the molecular mechanisms underlying the species-specific response of *Candida* against LPS during biofilm formation, and to evaluate the role of LPS as a putative antifungal agent.

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