MYCOLOGY

The effect of oral commensal bacteria on candidal adhesion to human buccal epithelial cells in vitro

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The effect of Streptococcus sanguis, S. salivarius, Escherichia coli and Porphyromonas gingivalis on the adhesion of Candida albicans and C. krusei to human buccal epithelial cells (BEC) was investigated with a modified membrane filter system. The filters (12 μm diameter pores) acted as a support for the BEC which were pre-exposed to known concentrations of bacterial suspensions (for 45 min–1 h), and then re-incubated with standardised concentrations of yeast suspensions for various periods. The BEC with adherent yeasts were then transferred on to a glass slide, gram-stained and counted by light microscopy. Three of the four bacterial species significantly suppressed adhesion of C. albicans to BEC; S. sanguis had no effect. Both S. sanguis and S. salivarius suppressed adhesion of C. krusei to BEC pre-exposed to three different bacterial concentrations, although variable results were obtained with P. gingivalis and E. coli. Significant differences in the relative adhesion of C. albicans and C. krusei to BEC were also recorded. These results indicate that the adhesion of yeasts to BEC is modulated both by the composition and the quantity of the pre-existing bacterial flora on the BEC.

Introduction

Adherence and colonisation on an epithelial surface are the first steps by which a micro-organism may initiate infection. Although there have been many studies of the adhesive mechanisms of oral bacteria and yeast species to epithelial cells [1–8] little attention has been paid to the possible effect of oral commensal bacteria on the adhesion of Candida albicans and other clinically important pathogenic yeasts, such as C. krusei, to epithelial surfaces. Makrides and MacFarlane [5] showed that while bacteria such as Escherichia coli and Klebsiella aerogenes enhance candidal adherence, others, including Streptococcus sanguis and S. mitis, decrease yeast adherence to epithelial cells. Very recent studies have also demonstrated the effect of indigenous bacterial populations on buccal epithelial cells (BEC) on subsequent candidal adhesion in vitro [9].

The present study attempted to extend previous investigations by observing the effect of four different oral bacterial species at three different concentrations on the subsequent adhesion of two Candida species – C. albicans and C. krusei – to human BEC.

Materials and methods

Preparation of buccal epithelial cells

Human BEC from six healthy adults (four males and two females; age range 25–45 years) were collected in the mornings, c. 1 h after breakfast, by gently rubbing the inner aspect of the cheek mucosa with two sterile wooden spatulas, and dispersed in 5 ml of phosphate-buffered saline (PBS, 0.5 M, pH 7.2). The BEC suspensions were pooled and washed four times in PBS to remove loosely attached organisms, by centrifugation at 3500 rpm for 10 min. The BEC were then resuspended to a concentration of 1.0 × 10^5 cells/ml by haemocytometer counting, and were used immediately for the adhesion assay.

Preparation of bacterial suspensions

Test strains of two species of oral streptococci, S. sanguis SK 142 and S. salivarius SK 56, received from the Royal Dental College, Aarhus, Denmark, were maintained on blood agar at 4°C and subcultured monthly. One half plate of an overnight blood-agar culture of each strain was inoculated into Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 18 h; cells were harvested by centrifugation (3500 rpm for 10 min), washed once in PBS and the deposit was resuspended in 5 ml PBS. This suspension was further diluted in PBS to yield a
concentration of $10^7$ cfu/ml estimated by spectrophotometry and confirmed by spiral plating (Spiral System, OH, USA) of the suspension on to blood agar to obtain a total viable count. Three different concentrations of each species were prepared by serial dilution to yield final concentrations of $10^6$, $10^5$ and $10^4$ cfu/ml which were confirmed as described above. Both methods of bacterial quantification yielded reproducible results.

*E. coli* ATCC 25922 was obtained from the American Type Culture Collection, Rockville, MD, USA, and was maintained on MacConkey agar plates at 4°C and subcultured monthly. A heavy inoculum of an overnight culture was grown in TSB at 37°C for 18 h. The cells were harvested by centrifugation, washed once and resuspended in 0.5 M PBS; final concentrations of $10^6$, $10^5$ and $10^4$ cfu/ml were prepared as described above.

*Porphyromonas gingivalis* Pg 50 was obtained from Dr M. Haapasalo, Department of Cariology, University of Helsinki, Finland and cultured on blood agar (Columbia agar base with horse blood and haemin) anaerobically at 37°C for 3–4 days and then transferred to 5 ml of TSB and re-incubated for another 3 days at 37°C under strict anaerobic conditions (CO$_2$ 10%, H$_2$ 10%, N$_2$ 80%) in an anaerobic chamber (Forma Scientific, Inc., USA). The cells were harvested by centrifugation, washed once and suspended in 0.5 M PBS for the assay. Standardised suspensions were prepared as described above.

**Preparation of Candida suspensions**

Representative strains of two species of *Candida* were tested. *C. albicans* GDH 18 and *C. krusei* GDH 88/1742, both isolated from patients with denture stomatitis, were obtained from the Oral Microbiology Laboratory, Glasgow Dental Hospital and School, Glasgow. Stock cultures were maintained on Sabouraud’s Dextrose Agar slopes (Oxoid) at 4°C and were transferred monthly. A loopful of stock culture was incubated on Sabouraud’s agar for 18 h at 37°C. Three-to-four standard loopfuls of this culture were then inoculated into Brain Heart Infusion Broth (BHI; Oxoid) and incubated for 18 h at 37°C. The cells were harvested by centrifugation (3500 rpm for 10 min) washed with 0.5 M PBS (pH 7.2) and a final yeast concentration in the suspending medium and their number of individual BEC on to the surface of the slide for observation and quantification, although some residual BEC were retained on the filter surface. The preparations were air dried, fixed with methanol, and stained with a modified Gram’s staining technique (15 s for each stain). The number of adherent yeasts was quantified by light microscopy at ×400 magnification. Fifty BEC were observed for adherent yeasts in a random manner; clumped, folded or overlapping BEC were excluded. Each assay was performed on three separate occasions with duplicate determinations each time.

The residual indigenous bacterial load on BEC after the washing procedure was monitored in preliminary studies. The effect of exposure of BEC to different concentrations of *S. salivarius* SK 56 and *E. coli* ATCC 25922 was also evaluated in the pilot studies to ascertain the relationship between the bacterial concentration in the suspending medium and their numbers on BEC.

**Electron microscopy**

BEC with the adherent bacteria and yeasts were filtered through a polycarbonate filter (Nucleopore, 0.2 μm pore size) with a sterile disposable syringe. The resultant residue collected on the surface of the filter was washed once with 10 ml of distilled water to remove the unattached bacteria and yeasts. The filters with the adherent cells were fixed in Karnosky’s fixative (60 min), washed three times with distilled water and mounted quickly on a stage, freeze-dried in liquid nitrogen and sputter-coated with gold. The

**Adhesion assay**

A modified assay procedure was developed to study the effect of pre-adherent bacteria on subsequent yeast adhesion to BEC. Equal volumes (0.5 ml) of BEC (1.0 × 10^5 cells/ml) and a known concentration of the test bacterial suspension in PBS, were added to a microtube, mixed gently and then incubated at 37°C for 1 h in a waterbath with gentle agitation at 40 rpm. The cells were then harvested on 12-μm pore polycarbonate filters (Costar, MA, USA) with suction. A bacteria-free suspension of BEC was included as the control. The vacuum pump of the filter manifold (Sartorius, SM16547) was then switched off, and the inlet and outlet of the manifold were sealed, after which 0.5 ml of the yeast suspension (10^7 cells/ml) was introduced into each well containing a filter with BEC pre-exposed to test bacteria; 0.5 ml of PBS was added to the control well.

The entire filter system was then placed in the water bath with the filter manifold above the water level, and incubated for another 60 min at 37°C. The manifold was then relocated and the filters were washed with 30 ml of PBS to remove the unattached yeast cells. Each filter was removed carefully with forceps and placed firmly on a glass slide with the BEC against the glass surface. After 10 s, the filter was removed gently, leaving the BEC adherent on the glass slide. This process resulted in transfer of ample numbers of individual BEC on to the surface of the slide for observation and quantification, although some residual BEC were retained on the filter surface. The preparations were air dried, fixed with methanol, and stained with a modified Gram’s staining technique (15 s for each stain). The number of adherent yeasts was quantified by light microscopy at ×400 magnification. Fifty BEC were observed for adherent yeasts in a random manner; clumped, folded or overlapping BEC were excluded. Each assay was performed on three separate occasions with duplicate determinations each time.
Adhesion of C. albicans to BEC

Microscopic observations

SEM and TEM studies with Candida spp., E. coli and S. salivarius showed some bacteria-candida interactions, albeit infrequently. For example, in the case of S. salivarius, the yeasts were mainly observed on the filter surface while bacterial cells were predominantly on the surface of BEC (Fig. 1A and D). Although direct streptococcal and candidal interactions occurred infrequently, as shown in Fig. 1C and E, E. coli cells appeared to serve as sites for candidal adhesion or were closely associated with the yeasts (Fig. 1B and F).

Adhesion of C. albicans to BEC

Exposure of BEC to higher concentrations of S. sanguis SK 142 did not suppress significantly the subsequent adhesion of C. albicans to BEC. However, the lowest concentration of bacteria did significantly suppress adhesion of C. albicans, an unexpected finding (Table 1). All three concentrations of S. salivarius SK 56 suppressed the subsequent adhesion of C. albicans GDH 18 to BEC (Table 1). The mean reductions in candida adhesion when exposed to 10^6, 10^5 and 10^4 cfu/ml bacterial suspensions were 59%, 28% and 63%, respectively, with a total overall reduction of 50% when the data were pooled (Table 2). The overall degree of adhesion suppression observed was similar to that of C. albicans (i.e., 53%).

E. coli ATCC 25922 suppressed the adhesion of C. albicans significantly when exposed to the highest and lowest concentrations of bacteria (Table 2), but at an intermediate concentration of 10^5 cfu/ml no significant change in adherence was recorded. P. gingivalis Pg 50 significantly reduced the adhesion of C. krusei to BEC (p < 0.0001) only at a concentration of 10^5 cfu/ml (Table 2); no significant reduction in candidal adhesion at two lower concentrations was observed as reported for C. albicans.

Comparisons between Candida and bacterial species

In general, the species that suppressed candidal adhesion most was S. salivarius and the degree of inhibition was similar for both yeast species (c. 50%).
Fig. 1. A. Scanning electron micrograph of BEC with adherent *S. salivarius* SK 56 and *C. albicans* GDH 18. Note the predominance of streptococci on the buccal cell and the yeasts lying on the filter surface. B. Scanning electron micrograph of *E. coli* ATCC 25922 (e) and *C. albicans* GDH 18 adhesion on a BEC (inset: higher magnification). C and D. Transmission electron micrographs of *C. albicans* GDH 18 (ca) and *S. salivarius* SK 56 (s) on BEC. Note the association of yeast cells and bacteria with BEC. E. Scanning electron micrograph of adherent *S. salivarius* SK 56 and *C. krusei* GDH 88/1742 on a BEC. F. Scanning electron micrograph of adherent *E. coli* ATCC 25922 and *C. krusei* GDH 88/1742 (ck) on a BEC.
The overall percentage reductions in adhesion of both Candida spp. by P. gingivalis (24 and 23%) were also almost identical, though differences were recorded at different concentrations. In contrast, enhancement of candidal adhesion was observed on two occasions due to the presence of either S. sanguis SK 142 or E. coli ATCC 25922 (Tables 1 and 2). Overall, the suppression of candida adherence to BEC observed did not necessarily correlate with the bacterial load in the incubation suspension.

When the relative adhesion of C. albicans and C. krusei to BEC were compared with pooled data from the studies with all four bacteria, C. albicans was significantly more adherent than C. krusei, irrespective of whether the BEC were exposed to the bacteria or not (p < 0.06 and <0.006, respectively). Thus, in the pooled control and test samples C. albicans adherence was 27.4% and 26.8% greater than C. krusei adherence to BEC, respectively, indicating consistent intra-species variation in adhesion. Nevertheless, the total overall reduction in candidal adhesion of 33-35% exerted by the test bacteria for either Candida spp. was very similar.
Discussion

Candida spp. are intra-oral commensals in 40–60% of human subjects [10] and the precise factors involved in the development of candidosis are still not clear. One feature which is thought to contribute to the latter phenomenon is the ability of the yeasts to attach to BEC and the modulation of such adherence by commensal bacteria [4]. There have been several reports on both the adherence suppression and promotion effects of oral bacteria on Candida spp., although only a few have studied in detail the effect of different concentrations of oral bacteria on BEC. Liljemark and Gibbons [4] reported suppression of C. albicans by human salivary bacteria, including oral strains of S. salivarius and S. mitis in a gnotobiotic mouse model and this was confirmed subsequently with an in-vitro HeLa cell model [11].

C. albicans is considered the most pathogenic Candida species, although others (such as C. krusei) are currently gaining importance as emerging pathogens, especially in compromised patient groups [12]. Hence, this study aimed to evaluate the effect of four oral bacterial species on the adhesion of these two Candida spp. to BEC. S. sanguis and S. salivarius are considered to be predominant oral mucosal inhabitants and are known to affect candidal colonisation [4]; E. coli – a transient oral commensal – grows profusely in the months of compromised patients; and the strict anaerobe P. gingivalis is a known periodontal pathogen and is isolated occasionally from mucosal surfaces [13].

A number of methods have been used to study adhesion of candida to BEC. These include studies with gnotobiotic animals [4], exfoliated epithelial cells [2] and inert surfaces such as denture acrylic [11]. However, none has addressed comprehensively the complex issue of bacterial interference with candidal adhesion in an in-vitro system, possibly due to the limitations of the methodology. The modified method used in the current investigation to study this phenomenon was found to be relatively reproducible, especially when the higher concentrations of bacteria were employed. The filter manifold system used for the assay permitted easy mounting and retrieval of the BEC with the attached bacteria and yeasts. There have been no reported studies on the effect of different concentrations of bacteria applied to BEC on subsequent candidal adhesion. The overall data show that although exposure of BEC to a bacterial concentration of $10^8$ cfu/ml invariably resulted in reduced candidal adhesion, surprisingly there was no direct correlation between bacterial concentration and candidal adhesion, except in the case of P. gingivalis and C. albicans. One reason for this lack of correlation may be the decreased sensitivity of the technique at higher dilutions of bacteria and another could be the variable interactions that can occur between bacteria and Candida spp. on epithelial cells as previously documented [6, 14, 15].

The present data, in addition to confirming the results of previous studies, indicate that the degree of S. salivarius colonisation on BEC is important in suppressing candidal colonisation. These results concur with earlier studies in gnotobiotic mice [4] and of the effect of bacteria on C. albicans adhesion to HeLa cells [3, 5]. On the contrary, de Miranda et al. [15] were unable to demonstrate either in-vitro or in-vivo (in an aerobic continuous-flow system and mouse model, respectively) suppression of C. albicans in the presence of S. salivarius. C. albicans adhered in greater numbers to denture acrylic surfaces in the presence of S. sanguis [16] but no such effect was demonstrable in studies with HeLa cells [5]. The latter workers have also reported that S. mitior and some strains of S. sanguis suppress C. albicans adhesion to HeLa surfaces.

Another commensal organism that has been used frequently in candidal adhesion studies is E. coli and these have yielded varied results [14, 17]. The present study showed suppression of both C. albicans and C. krusei adhesion by the ATCC strain of E. coli used. These results are similar to those of Balish and Philips [17] who reported resistance to candida infection in gnotobiotic mice possessing a monoflora of E. coli. However, Makrides and MacFarlane [14] found an increased rather than suppressed adherence of C. albicans to HeLa cells in the presence of E. coli and explained this as being due to type 1 fimbrae on bacterial cells and mannose-like bridging receptors on both the bacterial and yeast surfaces. Indeed, Centeno [6] has studied the difference in adhesion properties of C. albicans when pre-treated with piliate and non-piliate strains of bacteria on epithelial cells, and found an enhancement of candidal adhesion with piliate bacteria and no effect with non-piliate strains. He concluded that type 1 pili of bacteria, which are found on enterobacterial surfaces, were responsible for this effect. Although the ATCC strain of E. coli tested is piliate (unpublished data) it is not known whether it produces type 1 pili or not. Furthermore, it is well known that subculture of organisms results in reduction or loss of surface appendages and variations in adhesion properties [18], and these too may have contributed to the current observations.

The only strict anaerobe used in this study, P. gingivalis, is a putative periodontal pathogen. Lamont et al. [19] have shown that it adheres to and colonises the cell membranes and microvilli followed by internalisation of the bacteria into the epithelial cell cytoplasm without causing any damage to the gingival epithelial cells [20]. The time of incubation played a major role in the adhesion and internalisation of this organism, and Sandros et al. reported the adherence of P. gingivalis to epithelial cells after a 3-h incubation period [20]. As the current results were obtained after pre-incubation for 60–90 min, it seems likely that adherence without internalisation occurred.
The significant reduction in adhesion of *C. albicans* caused by *P. gingivalis* Pg 50 suggests that the latter may play a role in colonisation resistance to *C. albicans* in the gingival crevice. As *P. gingivalis* is not commonly present in subgingival plaque, the importance of the present results in the overall ecology of *Candida* spp. remains uncertain. Recent reports of the isolation of *C. albicans* from gingival pockets, especially in HIV-infected individuals [21, 22], begs the question whether periodontal anaerobes play a role in preventing *C. albicans* colonisation.

There are many studies on *C. albicans* adhesion to BEC [2, 14, 23–25] but only a few have investigated the adhesion of *C. krusei* [23–26]. All workers have reported significant intra-species variation in the ability of yeasts to adhere to BEC, which was also confirmed in this study. However, we were unable to demonstrate the very high degree of adhesion of *C. albicans* compared with *C. krusei* (e.g., 11-fold increase) demonstrated in previous studies [23–26]. The low adhesion activity of *C. krusei* is thought to be related to their low virulence potential, although the relatively high adhesion observed in the present study could be due to the potency of this particular strain. It is also interesting to note in the pooled data the similarity in the overall reduction in candidal adhesion (33–35%) observed with both *Candida* spp. This implies that the binding sites of both *C. albicans* and *C. krusei* to BEC may be similar, although some have proposed a different cell-wall composition for *C. krusei* [12, 27, 28]. Whether there are significant intra-species differences in relation to the parameters that have been investigated needs to be studied with many strains from each species, as the present study used only one isolate each of *C. albicans* and *C. krusei*.

In conclusion, the current results indicate that oral commensal bacteria impose a degree of colonisation resistance on yeast flora and the degree of such resistance offered is likely to vary depending on both the quality and the quantity of the resident indigenous bacteria. Further detailed studies with the model described should help asses the significance of such interactions in the oral cavity.

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