Effects of tea extracts on the colonization behaviour of *Candida* species: attachment inhibition and biofilm enhancement

Yi Wang,¹,* H. M. H. N. Bandara,¹ Deirdre Mikkelsen¹,² and Lakshman P. Samaranayake¹,*

**Abstract**

**Purpose.** We assessed the effects of four different types of tea extracts (green, oolong, black and pu-erh tea) on cellular surface properties (hydrophobicity and auto-aggregation) and the colonization attributes (attachment and biofilm formation) of four strains of *Candida albicans* and three strains of *Candida krusei*.

**Methodology.** The cellular surface properties were determined using spectrophotometry. The colonization activities were quantified using colorimetric viability assays and visualized using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

**Results.** The tea extracts, in general, reduced the hydrophobicity (by 8–66%) and auto-aggregation (by 20–65%), and inhibited the attachment of two *C. krusei* strains (by 41–88%). Tea extracts enhanced the biofilm formation of one *C. albicans* and two *C. krusei* strains (by 1.4–7.5-fold). The observed reduction in hydrophobicity strongly correlated with the reduction in attachment of the two *C. krusei* strains (*P*<0.05). The ultrastructural images of the tea-treated *C. krusei* biofilm cells demonstrated central indentations, although they remained viable.

**Conclusion.** The tea extracts have the ability to retard *C. krusei* adhesion to glass surfaces, possibly by reducing fungal cellular hydrophobicity, whilst paradoxically promoting biofilm formation. In practical terms, therefore, consumption of tea beverages appears to have a complex effect on oral candidal colonization.

**INTRODUCTION**

*Candida* spp. are oral inhabitants of approximately 50% of the human population [1]. These microbes are considered to be important opportunistic pathogens, as they frequently cause infections in compromised individuals, such as those being treated with chemotherapy, human immunodeficiency virus (HIV)-infected individuals [2] and immuno-suppressed organ transplant recipients [3]. In general, *Candida albicans* is the most common oral species, whilst others, such as *Candida glabrata, Candida tropicalis, Candida krusei* and *Candida guilliermondii*, are less abundant, though consistently isolated [1]. Indeed, in some communities, *C. krusei* is the most prevalent *Candida* species isolated from the oral cavity [4].

Upon gaining access to the oral milieu, *Candida* spp. colonize the mucosal surfaces and abiotic surfaces, such as prostheses, including dental implants, and survive essentially as biofilms, which in essence exhibit greater resistance to host defences and antifungal agents than their planktonic counterparts [2, 5, 6]. Auto-aggregation of *Candida* cells (blastoconidia), their co-aggregation with other oral micro-organisms, and their attachment to dental hard and soft tissues, are the prerequisites for successful colonization and biofilm formation [7–9]. It is well known that the attachment of micro-organisms to different surfaces (or to each other) involves surface physico-chemical interactions, such as the hydrophobic, electrostatic and steric [10–13]. For example, the attachment of oral streptococci to abiotic surfaces correlates well with their cell-surface hydrophobicity [14], as well as their cell-surface charge [15]. In the case of fungi, especially yeasts which do not possess cell-surface appendages and are therefore more similar to colloidal particles, physico-chemical interactions are likely to play an important role in attachment and biofilm formation [16].
Traditional therapies for microbial infection are currently being challenged due to their potential undesirable side-effects, as well as the emergence of antimicrobial resistance, particularly in biofilm-related diseases [17]. Natural chemicals are therefore of great interest, and they have been studied extensively as novel agents to prevent Candida infections. Tea (Camellia sinensis), after water, is the second most popular drink worldwide [18], and its impact on oral candidal colonization, including biofilm formation, has been little studied. To date, the majority of research on tea and its by-products has focused on its antimicrobial, including anti-candidal, activity [19–23]. Yet, the impact of tea on candidal attachment and biofilm formation has not been studied extensively. We previously reported that tea extracts exhibited the ability to prevent Streptococcus mutans from attaching and forming biofilms on different abiotic surfaces, due to a superficial coating of tea components on the bacterial surfaces [13]. Similar investigations, to the best of our knowledge, have yet to be performed with Candida spp., despite the fact that yeasts are key constituents of the oral microbiome and are often found to influence the colonization of other oral bacteria [8, 24].

Therefore, in this study, we hypothesized that tea extracts affect the colonization behaviour of Candida spp. in a physico-chemical manner, akin to that of S. mutans, as demonstrated previously [13]. The aims of the current study, therefore, were to determine *in vitro* the impact of the extracts from four commercial tea products (with increasing degrees of fermentation) on the attachment, biofilm formation and physico-chemical properties of seven different Candida strains belonging to C. albicans (four strains) and C. krusei (three strains). Furthermore, we aimed to correlate the physico-chemical properties and colonization potential of tea-treated Candida species.

**METHODS**

**Microbial cultures**

Four strains of *C. albicans* (strains 1, SF1, E1 200/5/92 and ATCC 90028), along with three strains of *C. krusei* (strains CamL 27B, CamL 37B and ATCC 6258), were used in this study. All Candida strains, except the ATCC strains, are clinical isolates, and were obtained from the Candida collection at the Oral Biscience Laboratories, Faculty of Dentistry, University of Hong Kong. All strains were maintained on Sabouraud dextrose agar (SDA; Sigma-Aldrich, USA) at 4 °C, and grown in Sabouraud dextrose broth (SDB; Sigma-Aldrich, USA) at 37 °C under agitation (150 r.p.m.) for 24 h. Microbial cell suspensions were prepared by centrifuging 20 ml of SDB cultures at 3000 g for 5 min. Thereafter, the pellets were washed with 150 mM PBS (2.7 mM KCl, 10 mM Na₂HPO₄, 17 mM KH₂PO₄ and 137 mM NaCl, pH 7.4; Sigma-Aldrich, USA), and resuspended in 20 ml PBS for all experiments, unless otherwise stated.

**Preparation of tea extracts**

Extracts of four commercial tea products, namely green tea, oolong tea, black tea and pu-erh tea (T2 Co. Ltd, Australia; country of origin of the tea leaves: China) were prepared by mixing each tea with distilled water at a 1/20 (w/v) ratio for 24 h [25] at 37 °C. Thereafter, the mixture was filtered, and the filtrate was evaporated under vacuum at 40 °C, freeze-dried and stored at −20 °C for further use. The temperature was kept at or below 40 °C in all of the steps, as a higher temperature would destroy tea polyphenols.

Stock solutions of the tea extracts were prepared by dissolving 100 mg of extract in 10 ml of PBS and then filter sterilizing it through a 0.2 μm filter.

**Antimicrobial susceptibility tests**

The minimal inhibitory concentrations (MICs) of the tea extracts against the *Candida* strains were determined using the micro-broth dilution method as previously described by James [26]. Briefly, 100 μl of each filter-sterilized tea extract solution (at a final concentration of 20 mg ml⁻¹) was subjected to double dilution in a microtitre plate, mixed with 100 μl of SDB containing suspended Candida cells (approximately 10⁴ c.f.u. ml⁻¹) and incubated at 37 °C for 24 h. Growth was determined by visually assessing the turbidity in the wells. In subsequent experiments, each strain was treated with tea extracts at the concentration below the lowest MIC value among all tea extracts, to make the studies comparable. According to the results of the antimicrobial susceptibility tests, a concentration of 10 mg ml⁻¹ was chosen for all of the tea extracts for use in all subsequent assays.

**Determination of cell-surface hydrophobicity**

The cell-surface hydrophobicity was determined using the bacterial attachment to hydrocarbon (BATH) method, as previously described by Wang et al. [27], with the following modifications. Briefly, cell suspensions containing dissolved tea extracts were adjusted to OD₅₉₅=1.0±0.2. Controls were prepared using PBS without tea extracts, and tea extract–PBS solution without Candida cells. A 3 ml aliquot of each sample was mixed with 1 ml of hexane and vortexed for 2 min. The mixture was allowed to phase-separate for 1 h. The OD₅₉₅ of the aqueous layer was measured before (A₀) and after (A) the addition of hexane. The cell-surface hydrophobicity was expressed as % of binding to hexane = (1−A/A₀)×100 %.

**Auto-aggregation assays**

Auto-aggregation measurements were performed as described by Wang et al. [27]. A volume of 1 ml of cell suspension (with or without tea extracts) was adjusted to OD₅₉₅=0.25±0.05 prior to incubation at 37 °C for 6 h. The OD₅₉₅ was measured before (A₀) and after (A) the incubation. The aggregation percentage was expressed as % of auto-aggregation = (1−A/A₀)×100 % [28, 29].
Preparation of glass beads
Glass beads (4 mm; Eureka Beads, Australia) were degreased by soaking in acetone for 1 h, washed with 0.1 M HCl for 30 min, and then 0.1 M NaOH for 30 min, and rinsed in distilled water for 30 min, prior to autoclaving. Sterilized beads were oven-dried overnight and then stored appropriately for use in attachment experiments (and biofilm formation assays).

Microbial attachment assays
Microbial attachment assays were performed on prepared glass beads (as described above), using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay [30]. Briefly, a glass bead with 100 µl of cell suspension (at 10^7 c.f.u. ml⁻¹; with or without tea extracts) was incubated in a single well of a microtitre plate at 37°C for 1 h with shaking at 80 r.p.m. After incubation, the bead was removed from the well, gently washed three times with PBS to remove loosely attached cells, placed in a well of another microtitre plate containing 79 µl PBS, 20 µl XTT solution (1 mg ml⁻¹; Sigma-Aldrich, USA) and 1 µl of freshly prepared menadione solution (0.4 mM; Sigma-Aldrich, USA), and further incubated in the dark for 3 h at 37°C. Thereafter, the bead was removed from the well and colour changes of the solution in the well were measured using a microtitre plate reader (Spectra Max 340 tunable microplate reader; Molecular Devices Ltd, Sunnyvale, CA, USA) at 492 nm.

Biofilm assays
Biofilm formation assays were performed on prepared glass beads using the XTT reduction assay [30]. Briefly, a glass bead with 50 µl of tea extract (at a sub-MIC final concentration) and 50 µl double-concentrated SDB culture (10^5 c.f.u. ml⁻¹) were incubated in the well of a microtitre plate at 37°C for 48 h with shaking at 80 r.p.m. Controls were prepared by using distilled water instead of tea extract solutions. After incubation, the beads were treated as described in the microbial attachment assay protocol described above.

Scanning electron microscopy (SEM) study
The biofilm structures, cell distributions and surface topography of tea-treated and untreated samples were visualized using SEM. The untreated and tea-treated Candida biofilms were grown on glass slides (1 cm × 1 cm) by immersing a slide in 10 ml SDB culture and incubating it in a Falcon tube at 37°C for 48 h. After incubation, the slides were washed in PBS, air-dried and fixed with 4% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in PBS. The fixed slides were treated as described in the SEM study protocol described above.

Confocal laser scanning microscopy (CLSM) study
The viability of tea-treated and untreated biofilm cells was assessed using CLSM. The untreated and tea-treated Candida biofilms were grown on glass cover slips (1 cm × 2 cm) as described in the SEM study protocol above. After incubation, the slides were stained with SYTO 9 dye and propidium iodide (LIVE/DEAD BacLight Bacterial Viability kit; Invitrogen, Eugene, OR, USA) [31] prior to visualization using a Nikon C2 confocal laser scanning microscope (Nikon Corp., Tokyo, Japan). The CLSM study was only undertaken for the non-fermented green tea extract and post-fermented pu-erh tea, in order to compare the effects of monomeric and polymeric tea polyphenols.

Statistical analysis
All assays were carried out in triplicate with independently grown cultures, and all values were expressed as mean±sd. A one-way ANOVA (Tukey’s comparison) was performed to compare the control and treatments in each assay. The relationship between the properties of cell surfaces and their colonization ability was determined using regression plots. All data expressed as percentage values were normalized by arcsine transformation. All analysis was conducted using SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level.

RESULTS
Antimicrobial susceptibility tests
The results of the antimicrobial susceptibility tests indicated that at 20 mg ml⁻¹ the tea extracts could not kill or inhibit any of the Candida spp. (data not shown). Therefore, a concentration of 10 mg ml⁻¹ (a non-lethal dose) was chosen and used for all of the tea extracts in all of the assays in order to ensure that the effects of the tea extracts on the properties and colonization behaviour of Candida spp. could be tested without either killing or inhibiting the cells.

Determination of cell-surface properties and colonization behaviour
The results of the cell-surface hydrophobicity assays are shown in Fig. 1. It was found that the tea extracts significantly reduced the cell-surface hydrophobicity of most of the tested Candida strains (P<0.05) by 8–66%, except for C. kru sei ATCC 6258 (P>0.05), whose hydrophobicity was not reduced by any of the tea extracts. Of all the tea extracts tested, the pu-erh tea extract was the most effective in reducing the hydrophobicity (P<0.05, reducing it by 27–66%).

Similarly to the results obtained for the hydrophobicity assays, the tea extracts significantly reduced the auto-aggregation of all Candida strains, in most of the cases (P<0.05) by 20–65%, except for C. kru sei ATCC 6258 (P>0.05) (Fig. 2).

The results from the microbial attachment assays indicated that the tea extracts could only inhibit the attachment to glass beads of the C. krusei strains 27B and 37B (P<0.05), by 41–88% (Fig. 3). However, the biofilm assays indicated that the tea extracts did not inhibit the biofilm formation of most of the Candida spp. (P>0.05), but enhanced biofilm production by 1.4–7.5-fold for C. albicans strain ATCC.
**Fig. 1.** The effect of the tea extracts on the cell-surface hydrophobicity of *Candida albicans* and *Candida krusei*. For each strain, the values labelled with dissimilar letters indicate significant differences in hydrophobicity due to different types of tea treatment (*P*<0.05). The statistical comparisons were based on arcsine-transformed data (*n*=3).

**Fig. 2.** The effects of the tea extracts on the auto-aggregation of *Candida albicans* and *Candida krusei*. For each strain, the values labelled with dissimilar letters indicate significant differences in auto-aggregation due to different types of tea treatment (*P*<0.05). The statistical comparisons were based on arcsine-transformed data (*n*=3).
90028 and C. krusei strains ATCC 6258 and 37B (P<0.05) (Fig. 4). C. krusei strain 37B was therefore selected for subsequent microscopy studies.

**Correlation between cell-surface properties and colonization behaviours**

The correlations between the changes in the cell-surface properties (hydrophobicity and auto-aggregation) and the changes in the colonization behaviours (attachment and biofilm formation) due to the tea extract treatments were determined using a regression plot (Fig. 5). A significant positive correlation was observed between the changes in cell-surface hydrophobicity and the changes in the attachment of C. krusei strains 27B (Fig. 5a) and 37B (Fig. 5b). Regression values (R²) of 0.724 and 0.799 were observed (P<0.05 for both strains), respectively, suggesting that the reduction in hydrophobicity could be a mechanism underlying the attachment inhibitory effect of the tea extracts on C. krusei (strains 27B and 37B). A similar correlation was not observed for other strains (P>0.05). No correlation was observed between auto-aggregation and attachment/biofilm formation for all strains (P>0.05).

**Microscopy**

The SEM and CLSM images of the untreated and tea-treated C. krusei 37B cells are shown in Fig. 6. Although multiple SEM micrographs and CLSM images were taken, only one representative micrograph or image is presented here per treatment (Fig. 6a–g). While there were no significant morphological differences observed between the tea extract-treated and untreated cells in the attachment assays (images not shown), a marked difference was observed for the tea-treated and untreated cells in the biofilms. Despite all of the treated and untreated cells being processed in the same manner to avoid sample preparation biases, the tea extract-treated biofilm cells appeared to form denser clusters, and they presented with a dent or pock mark in the middle of each cell (Fig. 6c, d). However, the untreated cells appeared to be intact and formed smaller clusters (Fig. 6a, b). As this phenomenon was observed with Candida treated with all of the tested tea extracts, only the images of green tea-treated cells are shown (Fig. 6). Furthermore, the live/dead CLSM images indicated that the pock-marked, indented cells treated with green tea were all viable (Fig. 6f), unlike those treated with the pu-erh tea extract, where a scant distribution of non-viable cells was observed within the biofilm matrix (Fig. 6g).

**DISCUSSION**

The results from the antimicrobial susceptibility tests indicated that none of the crude tea extracts evaluated exerted any antifungal effect on the tested Candida spp., as they appeared not to kill or inhibit the yeasts at a relatively high tea concentration (20 mg ml⁻¹). The choice of whole crude tea extracts instead of isolated tea compounds could be a
possible reason for our findings, which contradict those of recent research, where a strong fungicidal effect has been reported [16, 20, 21]. However, our data imply that the crude polyphenols or their derivatives in tea are unlikely to inhibit or kill oral Candida, due to their relatively low concentrations, as well as the high temperature they experience, which would also destroy the tea compounds [25]. Nevertheless, we demonstrate here that ordinary drinking tea is likely to alter Candida colonization of the oral cavity in a species- and strain-dependent manner.

The results obtained from the surface property assays showed that the four tea extracts reduced cell-surface hydrophobicity and the ability to auto-aggregate. One could speculate that the tea components present in these extracts may have affected the cell surfaces by binding physically or chemically to the yeast blastospore surface, thereby altering their cell-surface properties. We have previously observed this phenomenon for S. mutans, a major cariogenic organism commonly found in the oral cavity [13]. In that study, we observed that tea polyphenols (especially tannins) coating the cells of S. mutans suppressed their adhesion, as well as biofilm formation, on three different abiotic surfaces: glass, stainless steel and hydroxyapatite – the major constituent of tooth enamel. Furthermore, this veneer of ‘tea coating’ was also visible through electron microscopy. However, in the present study, no such visible alterations of the cell surfaces of C. krusei were observed after tea-extract treatment (Fig. 6).

As the tea components were extracted using water, it could be surmised that most of the extracts were highly polar and able to interfere with the hydrophobicity of the cell surfaces. According to interfacial thermodynamic theory [32], the reduced hydrophobicity may result in weaker hydrophobic interactions between the cells and the substratum surface, as well as between different cells, which in turn would reduce auto-aggregation and, thus, in theory, the attachment of the cells to the contact surface. The findings of our study fit this model very well, as we observed a very high correlation between reduction in hydrophobicity and attachment and tea-extract exposure ($R^2$ of 0.724 and 0.799, respectively).

However, this was not the case for the attachment of all the C. albicans strains tested, and for some strains of C. krusei. Clearly, hydrophobic interactions alone may not entirely explain the key mechanisms that mediate the attachment of these strains. Microbial attachment is often deemed to be a two-step process. Physico-chemical interactions usually dominate the initial step, helping the cells to approach the contact surface and attach loosely to it. In the second step, cell-surface proteins/adhesins play their role in helping the cells to stick firmly to the surface [33]. Hence, for those strains whose attachment was not affected by hydrophobic interactions, other physico-chemical factors, such as electrostatic interactions or non-physico-chemical factors, e.g. cell-surface adhesins, might play the major role in their attachment behaviour [34].
As for C. krusei strains 27B and 37B, the reduction in hydrophobicity eventuated by the tea-extract treatment correlated strongly with the reduction in attachment, indicating that hydrophobic interactions may be the key factor affecting their attachment. In other words, reducing the hydrophobic interactions in the system could possibly control the initial colonization of these C. krusei strains. However, the attachment of the type-culture C. krusei strain (ATCC 6258) was not affected by the tea extracts, suggesting that the yeast may have altered cell-surface components. It is known that type-culture strains, such as C. krusei ATCC 6258, which are repeatedly sub-cultured in the laboratory over a prolonged period, lose their cell-surface attributes in comparison to their wild-type counterparts [1]. This phenomenon may account for the disparate behaviour of C. krusei ATCC 6258 we observed.

As attachment is the first stage of biofilm formation, it would be reasonable to assume that inhibiting attachment would reduce biofilms. However, the results obtained from the biofilm assays in this study indicated otherwise. The tea extracts did not inhibit biofilm formation by the tested Candida strains, but rather enhanced biofilm formation for some strains (C. albicans strain ATCC 90028 and C. krusei strains ATCC 6258 and 37B), suggesting that there might be variables other than physico-chemical interactions affecting their biofilm formation, such as chemical and biological factors. It could be speculated that the tea extracts might have induced a chemical stress on the cells, thus compelling them to aggregate and form thicker biofilms as a protective mechanism against this stress [35], which is evident in the microscopic images where the tea extract-treated cells formed denser biofilms. However, this was not supported by the auto-aggregation assays conducted in this study, probably due to the different experimental conditions used in the auto-aggregation and biofilm assays, such as incubation period (6 and 48 h, respectively) and growth media (PBS and SDB, respectively). Furthermore, it has been reported that tea polyphenols inactivate proteasomal enzymes in Candida cells, and these enzymes are used by the cells to regulate metabolism and respond to environmental signals [36]. Interfering with the functions of these enzymes could affect cell behaviour, in terms of proliferation and forming biofilms. Evensen and Braun [36] also reported that inactivating these enzymes inhibited biofilm formation by Candida spp., but this was not the case here. A possible reason could be that the tea polyphenols used by Evensen and Braun were pure compounds at relatively high concentrations, while the present study used crude tea-leaf extracts.

Interestingly, the microscopic images showed tea extract-treated C. krusei blastospores with central indentations or pock marks, in spite of which the cells were viable, as observed by live/dead stain microscopy. One exception was the pu-erh tea-treated samples, where a few non-viable cells were observed in the biofilm. Such findings could be explained in terms of the undissociated protons from the polyphenol molecules within the tea extract, with these causing cellular energy depletion with an increased ratio of ADP/ATP. This, in turn, may have inhibited DNA synthesis or arrested the process of cellular proliferation at the anaphase, a phenomenon previously reported by Tan et al. [37]. Thus, it is plausible that the cells with indentations could not yet complete the division due to this arrested development. This phenomenon has been observed and reported by other researchers in different micro-organisms, such as Salmonella [37] and Escherichia coli [38].

In conclusion, the four extracts from green, oolong, black and pu-erh teas used in this study did not kill or inhibit the growth of the tested Candida strains, but they did inhibit the attachment of two strains of C. krusei to glass surfaces, possibly due to a reduction in cell-surface hydrophobicity. However, the biofilm development of three of the Candida strains tested was enhanced by the tea extracts. There was also a simultaneous morphological change in the biofilm cells of C. krusei, leading to the formation of a central indentation in the cell walls of each blastospore. Therefore, while tea consumption may not necessarily kill oral Candida spp., it is likely to affect the initial colonization of oral surfaces by species such as C. krusei, while simultaneously enhancing their biofilm development.
Future studies need to focus on purifying specific compounds from tea extracts and evaluating the effect of the isolated compounds on Candida biofilm formation by a larger variety of Candida species, while expanding our knowledge of the effects of specific tea components on oral candidal colonization. Further, the removal effects of tea extracts/components on preformed oral biofilms should be investigated in addition to studying biofilm prevention effects. Finally, the ADP/ATP ratio could be measured by bioluminescent assays in order to confirm the assumption of cellular energy depletion induced by the tea extracts.

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Conflicts of interest
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