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ORIGINAL ARTICLE

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

Running title: Tea modulates *Candida* colonization

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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 and confocal laser scanning microscopy.

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 folds). 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, though 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 colonisation.

Keywords: *Candida*; tea; cell surface hydrophobicity; microbial attachment; biofilm.

44 **Abbreviations:** HIV, human immunodeficiency virus; SDA, sabouraud dextrose agar; SDB,

45 sabouraud dextrose broth; BATH, bacterial attachment to hydrocarbon; OD, optical density;

46 XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide; SEM,

47 scanning electron microscopy; CLSM, confocal laser scanning microscopy.

48

Introduction

Candida spp. are oral inhabitants of approximately 50% of the human population [1]. These microbes are considered important opportunistic pathogens as they frequently cause infections in compromised individuals, such as those on chemotherapy and HIV-infected individuals [2], and organ transplant recipients on immunosuppressives [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 prosthesis, 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 (blastospores), their co-aggregation with other oral microorganisms, as well as 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 microorganisms to different surfaces (or to each other) involves surface physico-chemical interactions such as hydrophobic, electrostatic, and steric [10-13]. For example, 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 cases 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 the attachment and biofilm formation [16].

Traditional therapies for microbial infection are currently challenged due to their potential undesirable side effects, as well as emergence of antimicrobial resistance,

73 particularly in biofilm-related diseases [17]. Natural chemicals have therefore been of great
74 interest, and extensively studied as novel agents to prevent *Candida* infections. Tea (*Camellia*
75 *sinensis*), after water, is the second most popular drink worldwide [18] and its impact on oral
76 candidal colonisation, including biofilm formation, has been little studied. To date, the
77 majority of research on tea and its by-products has focused on its antimicrobial, including
78 anti-candidal activities [19-23]. Yet, the impact of tea on candidal attachment and biofilm
79 formation has not been extensively studied. We previously reported that tea extracts exhibited
80 the ability to prevent *Streptococcus mutans* from attaching and forming biofilms on different
81 abiotic surfaces, due to a superficial coat of tea components on the bacterial surfaces [13].
82 Similar investigations, to our knowledge, have yet to be performed with *Candida* spp. despite
83 the fact that yeasts are key constituents of the oral microbiome and are often found to
84 influence the colonization of other oral bacteria [8, 24].

85 Therefore, in this study, we hypothesized that tea extracts affected the colonization
86 behaviour of *Candida* spp. in a physico-chemical manner, akin to that of *Streptococcus*
87 *mutans*, as demonstrated previously [13]. The aims of the current study, therefore, were to
88 determine *in vitro* the impact of the extracts from four commercial tea products (with
89 increasing degree of fermentation) on: i) the attachment and biofilm formation; and ii) the
90 physico-chemical properties of seven different *Candida* strains belonging to *C. albicans* (four
91 strains) and *C. krusei* (three strains). Furthermore, we aimed to correlate the physico-
92 chemical properties with the colonization potential of tea-treated *Candida* species.

93

94 **Materials and methods**

95 *Microbial cultures*

96 Four strains of C. *albicans* (strain 1, SF1, E1 200/5/92 and ATCC 90028), along with
97 three strains of *C. krusei* (strain CamL 27B, CamL 37B and ATCC 6258) were used in this
98 study. All *Candida* strains, except the ATCC strains, are clinical isolates, and were obtained
99 from the *Candida* collection at the Oral Bisocience Laboratories, at the Faculty of Dentistry,
100 University of Hong Kong. All strains were maintained on sabouraud dextrose agar (SDA;
101 Sigma-Aldrich, USA) at 4°C, and grown in sabouraud dextrose broth (SDB; Sigma-Aldrich,
102 USA) at 37°C under agitation (150 r.p.m.) for 24 h. Microbial cell suspensions were prepared
103 by centrifuging 20 ml of SDB cultures at 3000 g for 5 min. Thereafter, the pellets were
104 washed with 150 mM PBS (2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, and 137 mM
105 NaCl, pH 7.4; Sigma-Aldrich, USA), and resuspended in 20 ml PBS for all experiments,
106 unless otherwise stated.

107

108 *Preparation of tea extracts*

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

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

117

118 *Antimicrobial susceptibility tests*

119 The minimal inhibitory concentrations (MICs) of the tea extracts against the *Candida*
120 strains were determined using the micro-broth dilution method as previously described by
121 James [26]. Briefly, 100 µl of each filter sterilized tea extract solution (at a final
122 concentration of 20 mg ml^{-1}) was subject to double dilution in a microtitre plate, mixed with
123 100 µl of SDB containing suspended *Candida* cells (approximately 10^4 c.f.u. ml^{-1}), and
124 incubated at 37°C for 24 h. Growth was determined by visually assessing the turbidity in the
125 wells. In subsequent experiments, each strain was treated with tea extracts at the
126 concentration below the lowest MIC value among all tea extracts, to make the studies
127 comparable. According to the results of the antimicrobial susceptibility tests, a concentration
128 at 10 mg ml^{-1} was chosen for all tea extracts, for use in all subsequent assays.

129

130 *Determination of cell surface hydrophobicity*

131 The cell surface hydrophobicity was determined using the Bacterial Attachment to
132 Hydrocarbon (BATH) method as previously described by Wang *et al.* [27], with the
133 following modifications. Briefly, cell suspensions containing dissolved tea extracts were
134 adjusted to OD_{595} = 1.0±0.2. Controls were prepared by using PBS without tea extracts, and
135 using tea extracts-PBS solution without *Candida* cells. A 3 ml aliquot of each sample was
136 mixed with 1 ml of hexane and vortexed for 2 min. The mixture was allowed to phase
137 separate for 1 h. The OD_{595} of the aqueous layer was measured before (A_0) and after (A) the
138 addition of hexane. The cell surface hydrophobicity was expressed as % of binding to hexane
139 $= (1 - A/A_0) \times 100$ %.

140

141 *Auto-aggregation assays*

142 Auto-aggregation measurements were performed as described by Wang *et al.* [27]. A
143 volume of 1 ml of cell suspension (with or without tea extracts) was adjusted to OD_{595} =
144 0.25±0.05 prior to incubation at 37°C for 6 h. The OD_{595} was measured before (A_i) and after
145 (A_f) the incubation. Aggregation percentage was expressed as % of auto-aggregation = $(1-$
146 $A_f/A_i) \times 100$ % [28, 29].

147

148 *Preparation of glass beads*

149 Glass beads (4 mm; Eureka Beads, Australia) were degreased by soaking in acetone
150 for 1h, washed with 0.1 M HCl for 30 min, followed by 0.1 M NaOH for 30 min, and rinsed
151 in distilled water for 30 min, prior to autoclaving. Sterilized beads were oven dried overnight
152 and thereafter appropriately stored for use in attachment experiments (and biofilm formation
153 assays).

154

155 *Microbial attachment assays*

156 Microbial attachment assays were performed on prepared glass beads (as described
157 above), using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
158 (XTT) reduction assay [30]. Briefly, a glass bead with 100 µl of cell suspension (at 10^7 c.f.u.
159 ml^{-1}; with or without tea extracts), was incubated in a single well of a microtitre plate at 37°C
160 for 1 h with shaking at 80 r.p.m.. After incubation, the bead was removed from the well,
161 gently washed three times with PBS to remove loosely attached cells, placed in a well of
162 another microtitre plate containing 79 µl PBS, 20 µl XTT solution (1 mg ml^{-1}; Sigma-
163 Aldrich, USA) and 1 µl of fresh prepared menadione solution (0.4 mM; Sigma-Aldrich,
164 USA), and further incubated in the dark for 3h at 37°C. Thereafter, the bead was removed
165 from the well and colour changes of the solution in the well were measured using a microtitre

166 plate reader (Spectra Max 340 tunable microplate reader; Molecular Devices Ltd, Sunnyvale,
167 CA) at 492 nm.

168

169 *Biofilm assays*

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

176

177 *Scanning electron microscopy (SEM) study*

178 The biofilm structures, cell distributions and the surface topography of tea treated and
179 untreated samples were visualized using SEM. The untreated and tea treated *Candida*
180 biofilms were grown on glass slides (1cm × 1cm) by immersing a slide in 10 ml SDB culture
181 and incubating in a falcon tube at 37°C for 48 h. After incubation, the slides were washed in
182 PBS, air dried and fixed with 4% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in PBS. The
183 fixed slides were washed again in PBS, frozen in a -80°C freezer and freeze dried [13]. The
184 slides were then gold-sputtered and examined using a field emission scanning electron
185 microscope (Carl Zeiss Inc., Oberkocken, Germany) at 15 kV and an 8.4 mm working
186 distance.

187

188 *Confocal laser scanning microscopy (CLSM) study*

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

197

198 *Statistical analysis*

199 All assays were carried out in triplicate with independently grown cultures, and all
200 values were expressed as mean ± standard deviation. A one way ANOVA (Tukey's
201 comparison) was performed to compare the control and treatments in each assay. The
202 relationships between cell surface properties and their colonization abilities were determined
203 using regression plots. All data expressed as percentage values were normalized by arcsine-
204 transformation. All analysis were conducted using the SPSS software (PASW Statistics 18;
205 SPSS Inc.) at a 95% confidence level.

206

207 **Results**

208 *Antimicrobial susceptibility tests*

209 The results of antimicrobial susceptibility tests indicated that the tea extracts at 20 mg
210 ml^{-1} could not kill or inhibit any of the *Candida* spp. (data not shown). Therefore, a
211 concentration of 10 mg ml^{-1} (a non-lethal dose) was chosen and used for all tea extracts in all

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. krusei* 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 ($p<0.05$), by 27-66% reduction in hydrophobicity.

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. krusei* 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. ($p>0.05$), yet enhanced the biofilm production by 1.4-7.5 folds for *C. albicans* strain ATCC 90028 and for *C. krusei* strains ATCC 6258 and 37B ($p<0.05$) (Fig. 4). *C. krusei* strain 37B was therefore selected for subsequent microscopy studies.

Correlations between cell surface properties and colonization behaviours

234 The correlation between the changes in cell surface properties (hydrophobicity and
235 auto-aggregation) and the changes in colonization behaviours (attachment and biofilm
236 formation), due to the tea extract treatments, were determined using a regression plot (Fig. 5).
237 A significant positive correlation was observed between the changes in cell surface
238 hydrophobicity and the changes in attachment of *C. krusei* strains 27B (Fig. 5A) and 37B
239 (Fig. 5B). Regression values (R^2) of 0.724 and 0.799 were observed respectively ($p<0.05$ for
240 both strains), suggesting that the reduction in hydrophobicity could be a mechanism
241 underlying the attachment inhibitory effect of the tea extracts on *C. krusei* (strains 27B and
242 37B). A similar correlation was not observed for other strains ($p>0.05$). No correlation was
243 observed between auto-aggregation and attachment/biofilm formation for all strains ($p>0.05$).

244

245 *Microscopy*

246 The SEM and CLSM images of the untreated and tea-treated *C. krusei* 37B cells are
247 shown in Fig. 6. Although multiple SEM micrographs and CLSM images were taken, only
248 one representative micrograph or image is presented here per treatment (Fig. 6, A-G). While
249 there were no significant morphologic differences observed between the tea extract-treated
250 and untreated cells in the attachment assays (images not shown), a marked difference was
251 observed for the tea-treated and untreated cells in the biofilms. Despite all treated and
252 untreated cells were processed in the same manner to avoid sample preparation biases, the tea
253 extract-treated biofilm cells appeared to form denser clusters, and presented with a dent or
254 pock mark in the middle of each cell (Fig. 6 C and D). However, the untreated cells appeared
255 to be intact and formed relatively smaller clusters (Fig. 6 A and B). As this phenomenon was
256 observed with *Candida* treated with all the tested tea extracts, only the images of green tea
257 treated cells are shown (Fig. 6). Furthermore, the live/dead CLSM images indicated that the

258 pock-marked, indented cells treated with green tea were all viable (Fig. 6F) unlike those
259 treated with the pu-erh tea extract, where a scant distribution of non-viable cells was
260 observed within the biofilm matrix (Fig. 6G).

261

262 **Discussion**

263 The results from the antimicrobial susceptibility tests indicated that none of the crude
264 tea extracts evaluated exerted any antifungal effect on the tested *Candida* spp., as they
265 appeared not to kill or inhibit the yeasts at a relatively high tea concentration (20 mg ml^{-1}).
266 The choice of whole crude tea extracts instead of isolated tea compounds could be a possible
267 reason for our findings, which contradict those of recent research, where a strong fungicidal
268 effect has been reported [16, 20, 21]. However, our data imply that the crude polyphenols or
269 their derivatives in tea are unlikely to inhibit or kill oral *Candida*, due to their relatively low
270 concentrations, as well as the high temperature that would also destroy the tea compounds
271 [25]. Nevertheless, we demonstrate here that ordinary drinking tea is likely to alter *Candida*
272 colonisation of the oral cavity in a species and strain dependent manner.

273 The results obtained from the surface property assays showed that the four tea extracts
274 reduced cell surface hydrophobicity and their ability to auto-aggregate. One could speculate
275 that the tea components present in these extracts may have affected the cell surfaces by
276 possibly binding physically or chemically to the yeast blastospore surface, thereby altering
277 their cell surface properties. We have previously observed this phenomenon for
278 *Streptococcus mutans*, a major cariogenic organism commonly found in the oral cavity [13].
279 In the latter study, we observed that tea polyphenols (especially tannins) coating the cells of
280 *Streptococcus mutans* suppressed their adhesion, as well as biofilm formation on three
281 different abiotic surfaces: glass, stainless steel, and hydroxyapatite - the major constituent of

282 tooth enamel. Furthermore, this veneer of `tea coating` was also visible through electron
283 microscopy. However, in the present study, no such visible alterations of the cell surfaces of
284 *C. krusei* were observed after tea-extract treatment (Fig. 6).

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

293 However, this was not the case for the attachment of all *C. albicans* strains tested, and
294 for some strains of *C. krusei*. Clearly, hydrophobic interactions alone may not entirely
295 explain the key mechanisms that mediate the attachment of these strains. Microbial
296 attachment is often deemed as a two-step process. Physico-chemical interactions usually
297 dominate the initial step, helping the cells to approach the contact surface and loosely attach
298 to it. In the second step, cell surface proteins/adhesins play their role in helping the cells to
299 firmly stick to the surface [33]. Hence, for those strains whose attachment could not be
300 affected by hydrophobic interactions, other physico-chemical factors, such as electrostatic
301 interactions or non-physico-chemical factors, such as cell surface adhesins, might play the
302 major role in their attachment behaviour [34].

303 As for *C. krusei* strains 27B and 37B, the reduction in hydrophobicity eventuated by
304 the tea extract treatment strongly correlated with the reduction in attachment, indicating that
305 hydrophobic interactions may be the key factor affecting their attachment. In other words,

306 reducing the hydrophobic interactions in the system could possibly control the initial
307 colonization of these *C. krusei* strains. However, the attachment of the type-culture *C. krusei*
308 strain (ATCC 6258) was not affected by the tea extracts, suggesting that the yeast may have
309 altered cell surface components. It is known that type culture strains, such as *C. krusei*
310 ATCC 6258, which are repeatedly sub-cultured in the laboratory over a prolonged period,
311 loose their cell surface attributes in comparison to their wild-type counterparts [1]. The latter
312 phenomenon may account for the disparate behaviour of *C. krusei* ATCC 6258 we observed.

313 As attachment is the first stage of biofilm formation, it would be reasonable to assume
314 that inhibiting attachment would reduce biofilms. Yet, the results obtained from the biofilm
315 assays in this study indicated otherwise. The tea extracts did not inhibit biofilm formation by
316 the tested *Candida* strains, but rather enhanced biofilm formation for some strains (*C.*
317 *albicans* strain ATCC 90028 and *C. krusei* strains ATCC 6258 and 37B), suggesting there
318 might be variables other than physico-chemical interactions affecting their biofilm formation,
319 such as chemical and biological factors. It could be speculated that the tea extracts might
320 have induced a chemical stress on the cells, thus impelling them to aggregate and form
321 thicker biofilms as a protective mechanism against this stress [35], which is evident in the
322 microscopic images where the tea extract treated cells formed denser biofilms. However, this
323 was not supported by the auto-aggregation assays conducted in this study probably due to the
324 different experimental conditions used in the auto-aggregation and biofilm assays such as
325 incubation duration (6h and 48h, respectively) and growth media (PBS and SDB,
326 respectively). Furthermore, it has been reported that tea polyphenols inactivate proteasomal
327 enzymes in *Candida* cells, and these enzymes are used by the cells to regulate metabolism
328 and respond to environmental signals [36]. Interfering with the functions of these enzymes
329 could affect cell behaviour, in terms of proliferation and forming biofilms. Evensen and
330 Braun [36] also reported that inactivating these enzymes inhibited biofilm formation by

331 *Candida* spp., but this was not the case here. A possible reason could be that the tea
332 polyphenols used by Evensen and Braun were pure compounds at relatively high
333 concentrations, while the present study used crude tea leaf extracts.

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

345 In conclusion, the four extracts from green, oolong, black and pu-erh teas used in this
346 study did not kill or inhibit the growth of the tested *Candida* strains, but inhibited the
347 attachment of two strains of *C. krusei* to glass surfaces, possibly due to a reduction in cell
348 surface hydrophobicity. However, the biofilm development of three of the *Candida* strains
349 tested was enhanced by the tea extracts. There was also a simultaneous morphological change
350 in the biofilm cells of *C. krusei,* leading to the formation of a central indentation in the cell
351 walls of each blastospore. Therefore, in practical terms, while tea consumption may not
352 necessarily kill oral *Candida* spp., it is likely to affect the initial colonization of oral surfaces
353 by species such as *C. krusei,* while simultaneously enhancing their biofilm development.

354 Future studies need to focus on purifying specific compounds from tea extracts and
355 evaluating the effect of the isolated compounds on *Candida* biofilm formation by a larger
356 variety of *Candida* species, to determine the effectiveness as well as to expand the current
357 knowledge on the effect of specific tea components on oral candidal colonization. Also, the
358 removal effects of tea extracts/components on pre-formed oral biofilms should be
359 investigated in addition to studying the biofilm prevention effects. Furthermore, the
360 ADP/ATP ratio could be measured by bioluminescent assays in order to confirm the
361 assumption of cellular energy depletion induced by the tea extracts.

362

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365

366 **Conflict of interest**

367 The authors declare no conflict of interest.

368

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372

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Figure Captions:

Fig. 1 The effect of the tea extracts on cell surface hydrophobicity of *C. albicans* and *C. 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 auto-aggregation of *C. albicans* and *C. 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).

Fig. 3 The effects of the tea extracts on attachment of *C. albicans* and *C. krusei* to glass surfaces (OD reading; n=3). For *C. krusei* 27B and 37B, the values labelled with dissimilar letters indicate significant differences in attachment due to different types of tea treatment ($p<0.05$). Significant differences in attachment to glass surfaces were not observed after tea treatment for the remaining strains.

Fig. 4 The effects of the tea extracts on biofilm formation by *C. albicans* and *C. krusei* on glass surfaces (OD reading; n=3). For each strain, the values labelled with dissimilar letters indicate significant differences in biofilm development after different types of tea treatment ($p<0.05$).

493 **Fig. 5** Correlation between cell surface hydrophobicity (arcsine-transformed) and attachment
494 (OD reading) upon tea treatments plotted using binomial regression. (A) *C. krusei* 27B and
495 (B) *C. krusei* 37B.

496

497 **Fig. 6** SEM micrographs of control (untreated) *C. krusei* 37B biofilm (A: at 2,000 ×
498 magnification; B: at 5,000 × magnification; scale bar: 20 μm), green tea extract treated *C.*
499 *krusei* 37B biofilm (C: 2,000 × magnification; D: 5,000 × magnification; scale bar: 20 μm);
500 and confocal laser scanning microscopic images of untreated *C. krusei* 37B biofilm (E; scale
501 bar: 50 μm), green tea extract treated *C. krusei* 37B biofilm (F; scale bar: 10 μm), and pu-erh
502 tea extract treated *C. krusei* 37B biofilm (G; scale bar: 10 μm). Red arrows indicate the cells
503 with indentations (live-dead stain; yellow blastospores/cells indicate dead cells as opposed to
504 the live cells which are green).

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