



# The effect of saliva or serum on *Streptococcus mutans* and *Candida albicans* colonization of hydroxylapatite beads

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## ABSTRACT

**Objective:** Several recent reports imply the possibility of cariogenicity of denture plaque containing *Candida albicans*. Hence the purpose of this study was to investigate the effects of salivary and serum pellicles on *C. albicans* and *Streptococcus mutans* colonization on hydroxylapatite beads.

**Methods:** The colonization of three isolates of *C. albicans* and two isolates of *S. mutans* was examined by the use of a bioluminescent adenosine triphosphate (ATP) assay based on the firefly luciferase–luciferin system.

**Results:** In the preliminary study, a good correlation was observed between the cell number and ATP amount of each isolate tested, and the results yielded a level of significance ( $P < 0.001$ ; Student's *t*-test), confirming the validity of this method. When the relative ATP content of the 48 h colonization of both isolates of *S. mutans* were compared, a saliva pellicle was significantly more effective in promoting bacterial colonization than either uncoated or serum pellicle (ANOVA;  $P < 0.01$ ). In contrast, in the case of colonization of *C. albicans* isolates, a serum pellicle was significantly more effective in promoting the colonization of *C. albicans* GDH 18 and GDH 19, than both uncoated specimens and saliva pellicle (ANOVA;  $P < 0.01$ ). Similar trends were observed with *C. albicans* GDH 16, though significant differences were not observed (ANOVA;  $P > 0.05$ ).

**Conclusion:** The results suggest that the mechanism involved in fungal colonization on hydroxylapatite (HAP) should be different from that of mutans streptococci. © 1997 Elsevier Science Ltd.

**KEY WORDS:** *Candida albicans*, *Streptococcus mutans*, Colonization, Saliva, Serum, Hydroxylapatite

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## INTRODUCTION

*Candida albicans* is frequently recovered from denture fitting surfaces which act as a reservoir of infection in *Candida*-associated denture stomatitis (syn. chronic atrophic candidiasis)<sup>1,2</sup>. Since successful candidal colonization of denture surfaces has been recognized as an important step in the pathogenesis of this condition<sup>3</sup>, a

number of reports have described their adherence, colonization and biofilm formation on denture materials<sup>4–11</sup>.

A few recent reports have indicated a possible relationship between *C. albicans* and/or denture plaque and caries, or root caries. Beighton et al.<sup>12</sup> have reported the relatively high prevalence of *Candida* spp. (58.5%) from 82 root caries lesions (*C. albicans*, 54.9%), and others have shown that the wearing of overdentures is often associated with a high risk of caries and progression of periodontal disease adjacent to the abutment teeth<sup>13–17</sup>.

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In addition, it is known that the demineralization of root surface occurs at a pH range of 4.3–5.0, since the buffers or acid gels in this range of pH have been used for the reliable *in vitro* models of root caries<sup>18–21</sup>. Furthermore, sucrose rinses are reported to cause pronounced decrease in pH of denture plaque within 40 min (neutral pH to 4.4–4.7)<sup>22</sup> and Zraggen and Graf found that the ‘resting’ pH of denture plaque decreased from neutral values to a range between pH 4.0 and 4.5 after four to seven days sucrose rinses twice daily<sup>23</sup>. Several *in vitro* studies have supported the high acidogenic potential of *C. albicans*<sup>24,25</sup>. We also obtained similar findings, when the yeasts were grown on denture materials with or without protein pellicle coats<sup>26,27</sup>. These reports imply the possibility of demineralization of root or tooth surfaces in association with denture plaque containing *C. albicans*. It is noteworthy that the cariogenicity depends not only on the terminal pH, but also on several other factors, including the ability of the organism to colonize the tooth surface<sup>28</sup>. However, little attention, so far, has been paid to the adherence or colonization of *C. albicans* on hydroxylapatite. Recently Cannon et al. have reported that *C. albicans* adhered to saliva-coated hydroxylapatite with a relatively high affinity<sup>29</sup>.

In the adherence or colonization of microorganisms on the cervical lesion of tooth or root surfaces, the surface should be coated not only with saliva but also with serum proteins from periodontal fluids. The latter is known to modify the adherence, growth, colonization or biofilm formation of *C. albicans* on host or inert surfaces. Therefore, to examine the contribution of serum to the fungal adherence or colonization should be important in both clinical and biological terms.

Thus the purpose of the present study was to analyse the role of salivary and serum pellicles on *C. albicans* and *Streptococcus mutans* colonization on hydroxylapatite beads. To achieve this aim, we have adapted a bioluminescent adenosine triphosphate (ATP) assay based on the firefly luciferase–luciferin system, which is known as a simple and convenient method for accurate enumeration of viable cells<sup>30</sup> and has been previously used for assessment of gram-positive and gram-negative bacterial biofilms<sup>31,32</sup>.

## MATERIALS AND METHODS

### Microorganisms and growth conditions

#### *Candida albicans*

*Candida albicans* GDH 16, GDH18 and GDH 19, oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and School were used in this study. The isolates were identified by a sugar assimilation test using the API 20C system (API Products, Biomerieux, Lyon, France) and ‘germ tube’ test<sup>33</sup>.

A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250 mM glucose and grown aerobically at 37°C<sup>34</sup>. After overnight culture, the yeast was harvested in the late exponential growth phase, washed twice with sterile distilled water and resuspended to a final concentration of 10<sup>5</sup> cells/ml using a haemocytometer<sup>26</sup>. All yeast cells suspended, remained in the blastospore phase.

### *Streptococcus mutans*

*Streptococcus mutans* NTCC 10449 and Ingbritt, provided by the Department of Oral Microbiology, Hiroshima University, were used in the study. These microorganisms were cultured and prepared for experiments according to Satou *et al.* (1987)<sup>35</sup>. Briefly, all strains were grown in trypticase soy broth (Difco, Detroit, USA) supplemented with 0.5% yeast extract (Difco, Detroit, USA; TSBY). The cells were harvested during the exponential growth phase by centrifugation at 1000 *g*, washed twice with sterile distilled water and resuspended in the same buffer. The cell suspensions were subjected to a low-intensity ultrasonic device to disperse bacterial aggregates<sup>36</sup>. The optical densities of the suspensions were measured in a 1.0 ml cuvette with a 1 cm light path, and the suspensions were adjusted to give an OD<sub>550</sub> of 0.3 (corresponding to 3.65 × 10<sup>8</sup> CFU/ml).

In a preliminary study, we compared the viability of fungal or bacterial cells washed by either sterile distilled water or by 100 mM phosphate-buffered saline (pH 6.8), and no significant differences in viability were detected using these two methods.

## SALIVA AND SERUM

Unstimulated whole saliva was collected on ice from five healthy donors and an equal amount from each donor was pooled. The saliva was clarified by centrifugation at 12000 *g* for 15 min at 4°C<sup>21</sup>. Human serum was purchased from Sigma Chemical Co. (St Louis, MO, USA). Whole saliva and serum were stored at –25°C before use<sup>34</sup>.

## ASSAY PROCEDURES

### Cell numbers and ATP (adenosine triphosphate) analysis

To examine the interrelation between yeast or bacterial cell numbers and ATP content, 100 µl of serially diluted yeast (10<sup>2</sup>–10<sup>8</sup> cells/ml) or bacterial (10<sup>6</sup>–10<sup>10</sup> cells/ml) suspensions were dispensed into a series of sterile glass tubes. Then 0.9 ml of the extraction reagent was added to the each well and allowed to react for 30 min in an

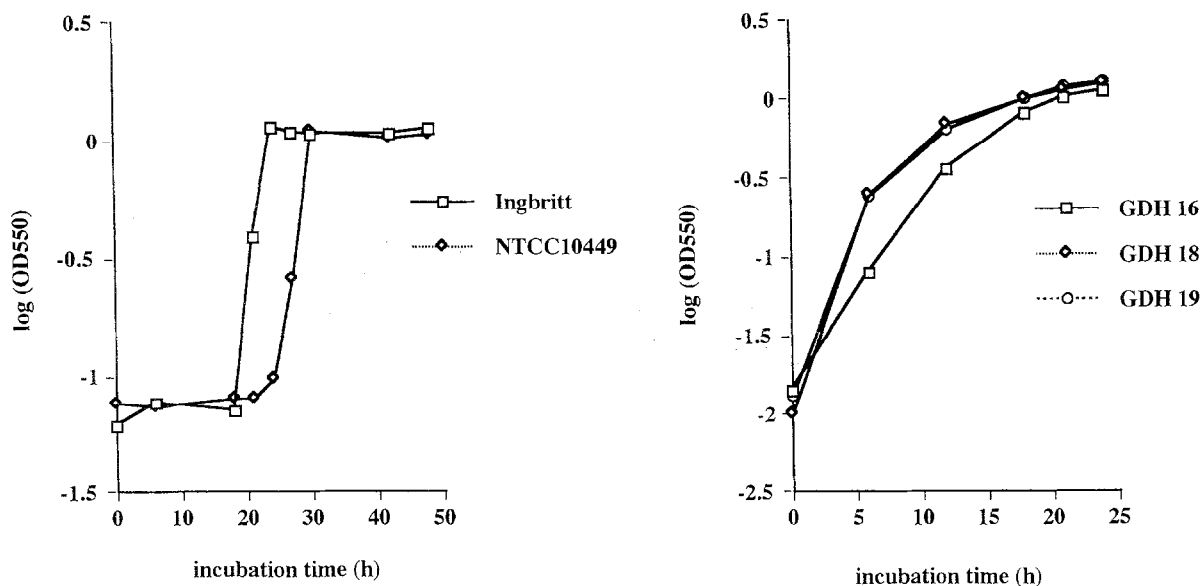


Fig. 1. Growth curve of each isolate of *S. mutans* and *C. albicans*.

ultrasonicator. The relationship between cell numbers and the amount of ATP was established using ATP analysis.

## GROWTH RATE OF MICROORGANISMS

As the growth rate of microorganisms should be important to assess the colonization of microorganisms, the growth rate of each isolate of *S. mutans* and *C. albicans* was examined by the change of optical density of the medium. Briefly, 100  $\mu$ l of yeast suspension ( $10^5$  cells/ml) or bacterial suspension ( $3.65 \times 10^8$  CFU/ml) was inoculated into 5 ml of Sabouraud broth (for yeasts) or TSBY (for streptococci) TSBY broth (for bacteria), and the changes in optical density at 560 nm of the media was monitored at 3 h intervals except for overnight. The growth rate (doubling time) of each isolate was calculated from the growth curve.

## COLONIZATION ASSAY

The colonization assay was conducted as follows. Four hundred and fifty microlitres of saliva or serum was dispensed into each well of Multiwell tissue culture plates (NuncloR Delta, Nunc, Kamstrup, Denmark); a Chemotaxicell (pore size 3  $\mu$ m; Kurabo, Osaka, Japan), into which 25 mg ( $24.96 \pm 0.07$  mg) of HAP beads were placed, was then inserted into the well. Subsequently, 150  $\mu$ l of saliva or serum were dispensed into each well of Chemotaxicell and incubated for 1 h at 37°C to allow formation of pellicle on the surface of HAP. Saliva or serum was substituted with an equal volume of sterile distilled water in the control wells. After incubation, 50  $\mu$ l of yeast ( $1 \times 10^5$  cells/ml) or bacterial suspension ( $3.65 \times 10^8$  CFU/ml) was inoculated into each well of Chemotaxicell and the whole assembly was incubated at 37°C for 2 h to promote

microbial adherence and colonization. Subsequently, 0.8 ml of Sabouraud broth (for yeasts) or TSBY (for streptococci) was carefully dispensed into each well, and incubated for 0, 24, 48, 96 and 168 h at 37°C. Afterwards each specimen was carefully removed, washed thoroughly by filtration through Watman No.41 filter-paper on three occasions with a total of 75 ml of distilled water to remove loosely adherent or uncolonized organisms. Then the HAP was immersed in 1.0 ml of the extraction reagent (benzalkonium)<sup>37</sup> and allowed to react for 30 min in an ultrasonicator. The amount of ATP was quantified using a bioluminescence apparatus (ATPA-100, TOA Electronics Ltd, Tokyo, Japan)<sup>31</sup>. This apparatus used the firefly luciferase system to determine the concentration of cellular ATP and is based upon the measurement of light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. In this system, the light intensity is directly proportional to the concentration of ATP<sup>37</sup>.

The assays were carried out on at least two independent occasions, with quadruplicated samples on each occasion. All the numerical data obtained were analysed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1% levels.

## RESULTS

### Growth rate of *S. mutans* and *C. albicans* isolates

The growth curves of streptococcal and fungal isolates are shown in Fig. 1.

The growth of each streptococcal and fungal isolate was plateaued within 24–33 h incubation and the growth rate of each isolate calculated from the growth curve was highest with *S. mutans* Ingbritt (doubling

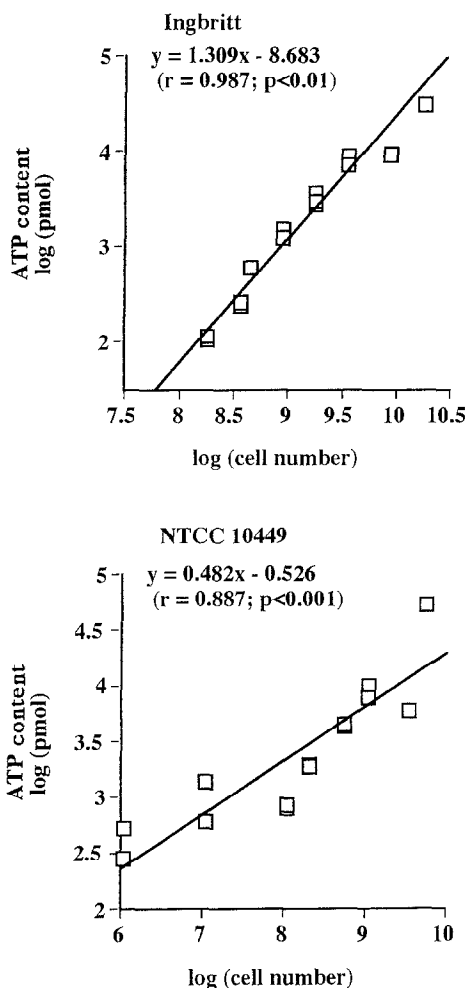


Fig. 2. Relationship between cell numbers and ATP content of *S. mutans* NTCC 10449 and Ingbritt.

time =  $1.50 \pm 0.17$  h), decreased in the order of *S. mutans* NTCC10449 (doubling time =  $1.71 \pm 0.17$  h), *C. albicans* GDH 18 (doubling time =  $1.96 \pm 0.45$  h), *C. albicans* GDH 19 (doubling time =  $2.13 \pm 0.47$  h), and *C. albicans* GDH 16 showed the least (doubling time =  $2.55 \pm 0.10$  h).

## RELATIONSHIP BETWEEN BACTERIAL AND YEAST CELL NUMBERS AND ATP CONTENT

As shown in Fig. 2, the cell numbers of both streptococcal isolates correlated well with the ATP content ( $r = 0.987$  for Ingbritt and  $r = 0.887$  for NTCC 10449;  $P < 0.001$ ; Student's *t*-test). Similarly, highly significant correlations were obtained with all three isolates of *C. albicans* (GDH 16,  $r = 0.988$ ,  $P < 0.001$ ; GDH 18,  $r = 0.996$ ,  $P < 0.001$ ; GDH 19,  $r = 0.991$ ,  $P < 0.001$ ; data not shown).

## COLONIZATION ON HAP

Colonization of HAP by *S. mutans* isolates reached a maximum after 48 h incubation then declined, irrespec-

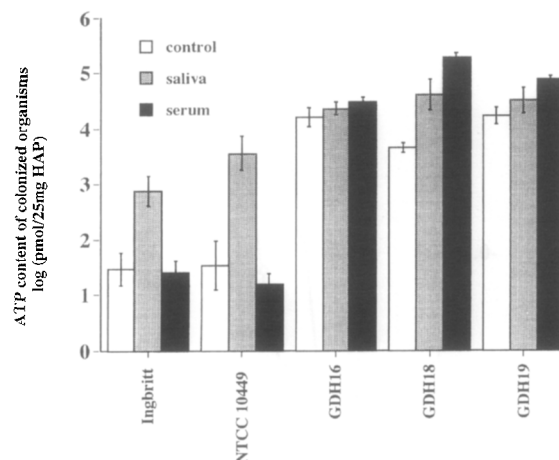


Fig. 3. Colonization of *S. mutans* and *C. albicans* on HAP beads.

tive of the presence of saliva or serum. In contrast, the colonization of each isolate of *C. albicans* gradually increased and plateaued after 72 h incubation. Thus, in Fig. 3, two days colonization of *S. mutans* and three days colonization of *C. albicans* are shown.

When the relative ATP content of the 48 h colonization of both isolates of *S. mutans* were compared, a saliva pellicle was significantly more effective in promoting bacterial colonization than either uncoated or serum pellicle (ANOVA;  $P < 0.01$ ). In the case of *S. mutans* Ingbritt, saliva-coated beads exhibited more than 10-fold more extensive colonization than the control (uncoated) and serum-coated beads. In the case of *S. mutans* NTCC 10449, the activity on the saliva-coated beads exceeded that of the controls or serum coated beads by a factor of 100.

When the relative ATP content of the 72 h colonization of *C. albicans* isolates were compared, a serum pellicle was significantly more effective in promoting the colonization of *C. albicans* GDH 18 and GDH 19, than both uncoated specimens and saliva pellicle (ANOVA;  $P < 0.01$ ). In the case of *C. albicans* GDH 18, the colonization on the serum-coated beads exhibited more than a 40-fold increase of cellular kinetics than the control, uncoated beads, and the activity on the saliva-coated beads exceeded that of the controls by a factor of 10. In the case of *C. albicans* GDH 19, the activity on serum-coated beads was four-fold higher than that of control and saliva-coated beads was two-fold higher than that of control. Similar trends were observed with *C. albicans* GDH 16, although significant differences were not observed (ANOVA;  $P > 0.05$ ).

When the ATP content of maximum microbial colonization was compared, the colonization of fungal isolates on both saliva- and serum-coated specimens was significantly higher than the colonization on saliva-coated HAP of either isolate of *S. mutans* (ANOVA;  $P < 0.05$ ). When the corresponding cell numbers of colonized microorganisms were calculated, *S. mutans* Ingbritt on saliva-coated HAP corresponded to approximately  $6.8 \times 10^8$  cells/25 mg, and the cell numbers

of colonization of *S. mutans* NTCC 10449 on saliva-coated HAP was approximately  $3.0 \times 10^8$  cells/25 mg, while the cell number of colonized yeasts on saliva and serum-coated beads corresponded to  $2.3 \times 10^5$  and  $3.0 \times 10^5$  cells (for GDH 16),  $4.1 \times 10^5$  and  $1.5 \times 10^6$  cells (for GDH 18), and  $3.1 \times 10^5$  and  $7.0 \times 10^5$  cells (for GDH 19) per 25 mg HAP, respectively.

## DISCUSSION

HAP beads coated with salivary components have been used previously to measure and to analyse the adherence of oral bacteria. However, there is only limited information available on the adherence of *Candida* to HAP, although various experimental approaches also have been made to examine the mechanisms of *C. albicans* adherence to solid surfaces, such as denture acrylic<sup>5-10</sup>.

Even the earliest investigators of this topic, using visual quantification of adherent yeasts, reported the high affinity of *C. albicans* to denture acrylic and modulation of this attachment process due to saliva and serum pellicles<sup>11,38</sup>. Although the latter groups observed the initial suppression of *C. albicans* adherence on saliva-coated denture surfaces<sup>9</sup>, others have reported the opposite<sup>10</sup>. While these may reflect the modulation of candidal adhesion due to variables such as the quality of the saliva<sup>39</sup>, the yeast isolates and the growth media, it should also be noted that the phenomenon of adherence may represent only the first step in the colonization process<sup>40</sup> which, as time progresses, leads to a formation of a thin biofilm and then a multilayer, climax community of plaque.

A deeper understanding of adherence and the subsequent behaviour of *Candida* colonizations or biofilms requires first, the examination of sequential samples over a period of time and more importantly, a method for accurate quantification of yeast biofilm formation which may exhibit dimorphic growth patterns as well as coadhesion, aggregation and multilayer growth over a prolonged period of colonization<sup>26</sup>. The main impediment for such research to date has been the lack of a relatively simple assay system to accurately quantify the cell growth. Recently, Hawser and Douglas reported fungal biofilm formation on catheter material by using dry weight, colorimetric (MTT) and radiometric assays<sup>41</sup>. However, in preliminary studies, we found that the dry weight and MTT assay methods were less sensitive in detecting the early stage of yeast colonization. Furthermore, the results of MTT assay were affected by the salivary and serum proteins (data not shown). Hence we adopted another assay system, bioluminescent ATP assay, which has been previously used to measure bacterial biofilm activity<sup>31,32</sup>. Farber and Wolff (1993) have conclusively shown that the result obtained by this ATP assay was consistent with that obtained by either conventional viable counts or radi-

olabelling methods. Using this method we noted an excellent correlation between the yeast cells and the ATP content in either the test or the control samples, as the results of our previous studies<sup>4,42,43</sup>. This was not surprising as the assay was based on the fundamental principles of ATP analysis which states that the amount of cellular ATP correlates with the dry weight, the volume and the number of viable cells<sup>37</sup>, irrespective of the morphological attributes of the yeast<sup>4</sup>.

Experiments with a firmly adsorbed pellicle are widely used to analyse the mechanisms of microbial adherence, but these may have some disadvantages to simulate only an incomplete and static picture of the oral environment, as microbial colonization and subsequent biofilm formation is a complex phenomenon with interactions not only between the pellicle proteins and the organisms but also the constituents of the solid-liquid interface such as the environmental saliva/serum which may modify phenomena such as coaggregation, coadhesion and clearance. Hence, in the present study, we used the Chemotaxicell system to analyse the role of both free and firmly adsorbed pellicle proteins. As the described ATP quantification method allows such studies, the colonization assay was carried out under conditions in which both adsorbed and free, saliva/serum proteins existed.

Particularly, the cervical regions of teeth surfaces or root surfaces are usually covered not only by saliva but also exudate or serum components derived from subgingival fluids. The latter have been reported to modify or enhance the fungal adherence or biofilm formation<sup>4,11</sup>, thus we examined the role of both saliva and serum proteins in the colonization of streptococci and fungi.

The amount of colonization of *S. mutans* isolates reached maximum after 48 h incubation then declined, irrespective of the presence of saliva or serum. In contrast, the colonization of *C. albicans* isolates gradually increased and plateaued after 72 h incubation. This phenomenon may be attributed to the differences in growth medium and the relative growth rate of organisms (*Fig. 1*).

When the relative ATP content of the 48 h colonization of both isolates of *S. mutans* were compared, a saliva pellicle was significantly more effective in promoting bacterial colonization than either uncoated or serum pellicle (ANOVA;  $P < 0.01$ ), concurring with many previous reports. The fact that serum-coated beads suppressed the colonization of streptococci could partly be attributed to the suppression of bacterial growth (data not shown).

When the relative colonization potential of *C. albicans* isolates were compared, a serum pellicle was significantly more effective in promoting the colonization of *C. albicans* GDH 18 and GDH 19, than both uncoated specimens and saliva pellicle (ANOVA;  $P < 0.01$ ). Similar trends were observed with *C. albicans* GDH 16, though significant differences were not

observed. These findings resemble the results of previous similar biofilm assays where acrylic substrates were used<sup>4</sup> and recent observations of Cannon *et al.* (1995) who observed the enhanced adherence of *C. albicans* to saliva-coated HAP as compared with uncoated HAP<sup>29</sup>.

When the ATP content of colonized microorganisms were compared, all fungal isolates colonized both saliva- and serum-coated specimens to a significantly higher degree than either isolate of *S. mutans*. Furthermore, the finding that serum coats promoted the fungal colonization on HAP should be important in clinical terms, since this implies that periodontitis or denture stomatitis should facilitate the fungal colonization on tooth surfaces. Whether this contributes to caries or root caries is still unexplained, because there should be the antagonism against *Candida* by bacteria in the oral cavity.

Our results, taken together suggest that *C. albicans* possesses the potential to colonize or form biofilms on HAP as much as *S. mutans* and the fact that serum potentiates the fungal colonization on HAP, implies that the mechanism involved in fungal colonization on HAP should be different from that of mutans streptococci.

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