Inhibitory effects of oral Actinomyces on the proliferation, virulence and biofilm formation of Candida albicans

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ABSTRACT

Objective: The pathogenesis of Candida-associated stomatitis involves the dysfunction of flora antagonistic to Candida. Oral Actinomyces species play an important role in regulating the oral microecological balance. The objective of this study was to investigate the antagonism of three oral Actinomyces against Candida albicans.

Design: Suspensions, culture supernatants and bacterial lysates of Actinomyces viscosus, Actinomyces naeslundii and Actinomyces odontolyticus were investigated for their actions upon six clinical strains of C. albicans were also tested. The proliferation of C. albicans was assessed using a liquid co-cultivation assay. The adhesion, acid protease and extracellular phospholipase activity, hyphae growth, and biofilm formation of C. albicans were measured.

Results: The results showed that the suspensions, culture supernatants and cell lysates of 10^6 colony forming units/ml oral Actinomyces significantly inhibited the proliferation of C. albicans (all P < 0.001). The culture supernatants exhibited significant antagonistic interactions in terms of adhesion (A. viscosus P < 0.001, A. naeslundii P = 0.016 and A. odontolyticus P = 0.009), acid protease (A. viscosus P = 0.035, A. naeslundii P = 0.022, A. odontolyticus P < 0.001) and phospholipase activities (A. viscosus P = 0.011, A. naeslundii P = 0.042, A. odontolyticus P = 0.021) of Candida, as well as its hyphae growth (A. viscosus P = 0.002, A. naeslundii P = 0.008, A. odontolyticus P = 0.006). Inhibition of C. albicans biofilm formation was also observed.

Conclusions: This study provides preliminary evidence that oral Actinomyces have inhibitory effects on the proliferation, adhesion, metabolic enzyme activity, hyphae formation and biofilm development of C. albicans.

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1. Introduction

Excessive antibiotic use and long-term denture wearing are the most common reasons for Candida-associated stomatitis, the pathogenesis of which still requires clarification. Some evidence suggests that it involves the dysfunction of oral flora antagonistic to Candida as the development of oral candidiasis sometimes occurs after long-term antibiotic use. Long-term broad-spectrum antibiotic therapy might kill or inhibit the flora antagonistic to Candida, resulting in excessive multiplication of Candida and the consequent development of antibiotic-associated stomatitis.1 In the oral microecological environment, some bacteria may inhibit the adhesion of Candida through their prior colonization of the surface of the denture. Reducing these antagonistic bacteria could cause massive growth or altered virulence of Candida, resulting in denture-associated stomatitis.2,3 Candida exerts its pathogenicity through its proliferation and via many kinds of biological virulence. The virulence of Candida predominantly includes adhesive forces, invasive enzymes, the formation of hyphae and biofilms, which play important roles in the establishment, adhesion and invasion of the oral mucosal surface. A stable biofilm will guarantee the growth and pathogenicity of Candida.4 The most common and most virulent species of Candida is Candida albicans.

The flora antagonistic to Candida have been studied since the early 1950s. At present, the focus is mainly on the antagonistic relationships between C. albicans and other oral bacteria, such as Lactobacillus, Pseudomonas aeruginosa and Streptococcus mutans. These studies have shown that the metabolites of Lactobacillus can inhibit the proliferation of C. albicans and destroy its internal structure.5 Pseudomonas aeruginosa inhibits the formation of hyphae and biofilms of C. albicans.6,7 Saliva streptococci play an antagonistic role mainly through their competitive nutritional requirements,8 although S. mutans not only inhibits colonization by Candida of different surfaces in the oral cavity, but also the formation of Candida biofilms.2,9 However, studies of these mechanisms have mainly focused on the molecular quorum-sensing system, such as 3-oxo-C12 homoserine lactone, a cell-cell signalling molecule produced by Pseudomonas aeruginosa, which was sufficient to inhibit C. albicans hyphae formation.10 While the full range of flora that inhibit C. albicans, their mechanisms of effects have not been determined yet.

Actinomyces species, which comprise part of the resident microflora of the oral cavity, constitute a considerable proportion of the normal human microflora and play an important role in regulating the oral microecological balance.11 Some research has shown that the colonization of the surfaces of teeth and the mucosa by Actinomyces increases the numbers and variety of plaque bacteria and encourages interactions between them.12 Actinomyces species can produce some anti-fungal material, including nikkomycin and geldanamycin.13 Therefore, we speculated that oral Actinomyces could establish an antagonistic relationship with oral Candida.

The aim of this study was to investigate the inhibitory effects of oral Actinomyces on C. albicans in terms of proliferation, adhesion, acid protease and phospholipase activities, hyphae growth and biofilm formation, and to make a preliminary assessment of the source of the inhibitory material. Clinical strains of oral C. albicans were used to verify the results.

2. Materials & methods

2.1. Strains and media

Candida albicans (American Type Culture Collection [ATCC] 1069), Actinomyces viscosus (ATCC 19246), Actinomyces naeslundii (ATCC 12104) and Actinomyces odontolyticus (ATCC 17929) were provided by the State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, Sichuan, China. Tryptone yeast extract (TPY) agar medium and TPY liquid medium were prepared for microbiological culture.

2.2. Microbiological fraction preparation

Samples were prepared according to a published method.14 C. albicans, A. viscosus, A. naeslundii and A. odontolyticus were cultured in TPY agar medium under aerobic and facultatively anaerobic conditions (80% O2, 10% H2, 10% CO2) at 37°C for 48 h, and then cell suspensions (2.5 × 10^6 colony-forming units [CFU]/mL) were prepared.

The culture supernatants of A. viscosus, A. naeslundii and A. odontolyticus were obtained by filtering part of the cell suspension through a 0.22 μm sterile filter (Millipore, Ireland). Suspension cultures of the three oral Actinomyces (2.5 × 10^8 CFU/mL) were collected by centrifugation at 2200 × g for 20 min and then ultrasonicated at 10 Hz in an ice bath at 4°C, with 30 s pulses, until the bacterial cells were completely lysed. The bacterial lysates were then collected by filtering the lysed suspensions through 0.45 μm sterile filters (Millipore, Ireland).

2.3. Clinical strains of C. albicans

Six clinical strains of C. albicans were isolated by cultivation from the saliva of six patients diagnosed with oral candidiasis on their first visit to the West China Hospital of Stomatology, Sichuan University, between June 2011 and March 2013. This study was conducted in accordance with ethical principles and was approved by the Ethics Committee of West China Hospital of Stomatology, Sichuan University. All the patients signed an informed consent before the initiation of research. The strains were identified as C. albicans by growth on the CHROMagar Candida selective medium (Zhengzhou Biocell Biotechnology Co. Ltd, Zhengzhou, China), stored at –40°C and recovered on TPY agar for 24 h at 37°C. Cells growing in this selective medium were considered as Candida species.15,16

2.4. Effects of oral Actinomyces on the proliferation of C. albicans

The effects of Actinomyces on C. albicans were evaluated according to a published method.17 Individual suspensions (1 mL) of A. viscosus, A. naeslundii and A. odontolyticus (2.5 × 10^6 CFU/mL) were added to suspensions of C. albicans
(2.5 × 10⁸ CFU/mL, 1 mL), as the experimental groups (we described them in the form of Av [A. viscosus] + CA [C. albicans], Ao [A. odontolyticus] + CA, An [A. naeslundii] + CA). To guarantee equal volume, an aliquot (1 mL) of the suspension culture of C. albicans was added to phosphate buffered saline (PBS) (1 mL) to produce the control groups. The groups for the culture supernatants and bacterial lysates of three oral Actinomyces on C. albicans were same as for the suspensions experiment. The three experimental groups and one PBS control group were cultured continuously for 120 h, and quantitative culture medium was added to ensure the supply of nutrition every 24 h. Aliquots (100 μL) of samples from each group were diluted to 1 × 10⁵ CFU/mL every 24 h for 5 days. A 20 μL diluted sample from each group was then inoculated onto CHROMagar selective culture medium (Zhengzhou Biocell Biotechnology Co. Ltd, Zhengzhou, China) and the colonies were counted after 24 h. Three parallel samples were established for each group and the experiment was performed in triplicate.

2.5. Effects of oral Actinomyces on the adhesion of C. albicans

Buccal epithelial cells (BECs) were isolated from frozen tissues from four healthy individuals (aged 24–26 years) according to Zeng et al. and Ellepola et al. pooled in 10 mL of PBS (pH 7.2) and washed twice by centrifugation at 2200 × g for 10 min. The cell concentration was then adjusted to 1 × 10⁵ cell/mL. Individual supernatants (0.5 mL) of A. viscosus, A. naeslundii and A. odontolyticus (2.5 × 10⁸ CFU/mL) were added to 0.5 mL of BEC suspension and 0.5 mL of C. albicans suspension (2.5 × 10⁶ CFU/mL). The control samples contained 0.5 mL of BEC suspension, 0.5 mL of C. albicans suspension and 0.5 mL of PBS. The experiment included three samples for each group and was performed in triplicate. The samples were incubated at 37 °C for 1 h with shaking at 75 rpm. The mixtures were then serially diluted, filtered (12 μm filter; Millipore), and the BEC/yeast cells were placed on a glass slide, air-dried and Gram-stained. The number of C. albicans cells adhering to 50 BECs was counted under a light microscope (Eclipse E100, Nikon Corp., Tokyo, Japan) at a magnification of 400× and recorded as the mean number of adhesions.

2.6. Effects of oral Actinomyces on the acid protease activity of C. albicans

The acid protease activity was measured based on the methods of Gokce et al. and Dagdeviren et al. Bovine serum albumin (BSA) agar medium (60 mL of a solution containing 4.0 g glucose, 0.2 g dried yeast extract, 0.5 g BSA, 0.04 g MgSO₄·7H₂O, 0.5 g KH₂PO₄, 1.0 g NaCl, 2.0 g agar [pH 4.0]) was sterilized. In the three experimental groups, the supernatant (1 mL) of each of the three oral Actinomyces was added to 1 mL of C. albicans suspension (2.5 × 10⁶ CFU/mL). A mixture of 1 mL of C. albicans suspension (2.5 × 10⁶ CFU/mL) and 1 mL of PBS buffer was prepared as the control. Each group was inoculated in triplicate. A 20 μL mixture of each sample was inoculated onto the BSA plates in duplicate at the same time and the plates were incubated at 37 °C for 7 days. The experiment was repeated three times and the average values were recorded. The acid protease activity was measured by the clearing of the opacity of the plates around the culture, the opacity was a measure of the precipitated albumin, the lower the precipitated albumin value, the stronger the acid protease activity.

2.7. Effects of oral Actinomyces on the phospholipase activity of C. albicans

Phospholipase activity was also based on Gokce et al. and Dagdeviren et al. egg yolk (2%) was added to sterilized Sabouraud dextrose agar (SDA) medium (100 mL of a solution containing 2.0 g malt agar, 1 M NaCl, 0.005 M CaCl₂ [pH 4.3]). The experimental groups and the control group were the same as those used for the acid protease test. A precipitation zone around the colony, which is an indicator of phospholipase activity, was observed. The phospholipase activity was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone. The experiment was repeated three times.

2.8. Effects of oral Actinomyces on the hyphae formation of C. albicans

The experimental groups and the control group were the same as those used for the acid protease test, and a liquid medium of RPMI 1640 + 10% foetal bovine serum was added to each sample. Each group included three samples, and the experiment was repeated three times. All samples were cultured for 48 h. The number of C. albicans germ tubes was counted in ten random fields and the means were calculated from 10 different randomly selected microscope fields.

2.9. Effects of oral Actinomyces on the biofilm formation of C. albicans

The effects of oral Actinomyces on the growth of C. albicans biofilms were evaluated according to the method of Rajendran et al. on sterile cover glass placed into the wells of a six-well microtitre dish (Corning). The experimental groups and control group were the same as those used in the acid protease test and were cultured for 48 h. The culture medium was then removed, and the coverslips were twice flushed carefully with PBS and then fixed in 2.5% glutaraldehyde for 24 h at 4 °C. The biofilms were then dehydrated in a graded series of 30%, 50%, 75%, 85%, 95% and 100% ethanol, changed every 15 min. The biofilms were observed with scanning electron microscopy (SEM; FEI/Holland). Each group included three samples and the experiment was repeated three times.

2.10. Effects of oral Actinomyces on the multiplication and virulence of clinical C. albicans strains

Individual suspensions (1 mL) of A. viscosus, A. naeslundii and A. odontolyticus (2.5 × 10⁸ CFU/mL) were added to each individual C. albicans clinical strain (2.5 × 10⁸ CFU/mL, 1 mL), as the experimental groups. An aliquot (1 mL) of the suspension culture of each clinical C. albicans strain was added to PBS (1 mL) to produce the control groups. After continuous culture for 120 h, the colonies were counted using the method described above. The six clinical C. albicans strains were tested respectively.
The groups and the experimental methods used to evaluate the effects of *A. viscosus*, *A. naeslundii* and *A. odontolyticus* on the virulence (including adhesion, acid proteinase activity, phospholipase activity and hyphae formation) and biofilm formation of the clinical *C. albicans* strains were the same as those described above.

2.11. **Statistical analysis**

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Because the frequency of colony count had a skewed distribution and each observed value a multiple relationship (geometric relation) and only had a normal distribution after positive logarithmic transformation, we recorded the colony count as an exponential (to base 10). The effects of the three oral *Actinomyces* on the proliferation activity of the ATCC type strain or clinical strains of *C. albicans* were analyzed using multivariate tests and Mauchly’s test of sphericity. The effects of the three oral *Actinomyces* on the adhesion, acid proteinase and phospholipase activities and hyphae formation of the ATCC type strain and clinical strains of *C. albicans* were analyzed with a test for the homogeneity of variances and analysis of variance (ANOVA). A probability value of <0.05 (two-sided) was considered as statistical significance.

3. **Results**

3.1. **Effects of oral *Actinomyces* on the proliferation of *C. albicans***

The number of *C. albicans* colonies decreased gradually as the concentration of the cell suspensions of the three oral *Actinomyces* and the time of co-culture increased to 120 h (Fig. 1). A significant reduction in *C. albicans* colonies was observed when liquid medium containing 10^6 CFU/mL oral *Actinomyces* was added. Compared with the control group, the numbers of live *C. albicans* colonies decreased significantly when it was co-cultured continuously in vitro with the suspensions (*P_{An+CA} < 0.001, P_{An+CA} < 0.001, P_{Ao+CA} < 0.001*), culture supernatants (*P_{An+CA} < 0.001, P_{An+CA} < 0.001, P_{An+CA} < 0.001*) or bacterial lysates (*P_{An+CA} < 0.001, P_{An+CA} < 0.001, P_{An+CA} < 0.001*) of each of the three oral *Actinomyces* at 48 h.

3.2. **Effects of oral *Actinomyces* on the virulence of *C. albicans***

As the culture supernatants effectively reduced the proliferation of *C. albicans* and were easily to be collected, they were then selected in the following experiments to observe the effect of oral *Actinomyces* on the virulence of *C. albicans*.

The virulence test results are shown in Table 1. The mean numbers of *C. albicans* cells adhering to BECs in the three experimental groups were *Av* + *CA* (1.216 ± 0.055), *An* + *CA* (1.318 ± 0.108) and *Ao* + *CA* (1.307 ± 0.064), all of which were significantly reduced (*P_{Av+CA} < 0.001, P_{An+CA} = 0.016 and P_{Ao+CA} = 0.009*) relative to the control group (1.427 ± 0.120). Although there was no significant difference between the effects of *A. naeslundii* and *A. odontolyticus* (*P = 0.797*), the adhesion allowed by the *A. viscosus* group was significantly different relative to the *A. naeslundii* group (*P = 0.023*), and the difference between *A. viscosus* and *A. odontolyticus* was also significant (*P = 0.041*).

The acid protease activities in the three experimental groups were *Av* + *CA* (0.694 ± 0.013), *An* + *CA* (0.697 ± 0.017) and *Ao* + *CA* (0.726 ± 0.035), and these were significantly different (*P_{Av+CA} = 0.035, P_{An+CA} = 0.022 and P_{Ao+CA} < 0.001*) from the control group (0.671 ± 0.019). The acid protease
activity of the *A. odontolyticus* group was lower than that of the *A. viscosus* group (*P* = 0.006) or the *A. naeslundii* group (*P* = 0.01). The activity of the *A. viscosus* group increased more than that of the *A. naeslundii* group did, although this difference was not statistically significant (*P* = 0.835).

The phospholipase activities of the three experimental groups were *Av* + *CA* (0.727 ± 0.044), *An* + *CA* (0.717 ± 0.030) and *Ao* + *CA* (0.722 ± 0.027), respectively, and the three activities were significantly higher (PAv+CA = 0.011, PAAn+CA = 0.042 and PAo+CA = 0.021) than that of the control group (0.682 ± 0.035). However, the activities of the three experimental groups were not significantly different (*P* > 0.5).

The mean numbers of hyphae in the three experimental groups were *Av* + *CA* (11.722 ± 1.457), *An* + *CA* (12.033 ± 1.679) and *Ao* + *CA* (11.989 ± 1.376), respectively, which were significantly reduced (PAv+CA = 0.002, PAAn+CA = 0.008 and PAo+CA = 0.006) relative to that of the control group (14.033 ± 1.419). However, the differences between the experimental groups were not statistically significant (*P* > 0.5).

### 3.3. Effects of oral *Actinomyces* on biofilm formation of *C. albicans*

As shown by SEM, less biofilm was formed by *C. albicans* in the experimental groups (*Av* Supernatant + CA group) than in the control group, which provides preliminary evidence that oral *Actinomyces* inhibits the formation of biofilm by *C. albicans* (Fig. 2).

### 3.4. Effects of oral *Actinomyces* on the proliferation and virulence of clinical *C. albicans* strains

The mean numbers of live colonies in the six experimental groups with clinical *C. albicans* strains were strongly reduced compared with the control group (PAv+CA < 0.001, PAAn+CA < 0.001 and PAo+CA = 0.003) when suspensions of the three oral *Actinomyces* were co-cultured with six clinical *C. albicans* strains, respectively. However, the differences among the experimental groups were not statistically significant (*P* > 0.5).

In the test of adhesion activity, there were significant differences between the three experimental groups and the control group of clinical *C. albicans* strains (PAv+CA = 0.002, PAAn+CA = 0.002 and PAo+CA = 0.004). Similar results were observed in the test of acid proteinase activity (PAv+CA < 0.001, PAAn+CA = 0.005 and PAo+CA = 0.002). Interestingly, in the test of phospholipase activity, there was a significant difference when the supernatant of *A. viscosus* was added (PAv+CA = 0.012), although there was no significant difference when the *A. naeslundii* and *A. odontolyticus* supernatants were tested (PAAn+CA = 0.602 and PAo+CA = 0.154). In the test of filament formation, there was no difference between the *A. viscosus* experimental group and the control group (PAv+CA = 0.063), but differences were observed between the *A. naeslundii* and *A. odontolyticus* groups and the control (PAAn+CA = 0.033 and PAo+CA = 0.031, respectively). Moreover, inhibitory effects of the oral *Actinomyces* on *C. albicans* biofilm formation were observed with SEM (data not shown).

There were no significant differences between the mean value of six clinical strains and the ATCC type strain of *C. albicans* either in the proliferation test (*P* = 0.534) or in the tests of the three virulence activities (PAdhesion = 0.163, PAacid protease = 0.417, PPhospholipase = 0.258 and PHyphae = 0.46). Therefore, the experiments with the clinical *C. albicans* strains confirmed the inhibition of the standard *C. albicans* strain by the three oral *Actinomyces*.

### 4. Discussion

The aim of this study was to investigate the inhibitory effects of oral *Actinomyces* on the proliferation, virulence, and biofilm formation of *C. albicans*. We found that three *Actinomyces*, *A. viscosus, A. naeslundii* and *A. odontolyticus*, significantly inhibited *C. albicans* proliferation. This inhibitory effect was demonstrated by the cell suspension, culture supernatant and bacterial lysate. The culture supernatant was also effective in antagonizing the virulence of *C. albicans* in terms of adhesion, acid protease and phospholipase activities, and hyphae formation.

In this study, we mainly focused on the number of *C. albicans*, and the inhibitory effects of the supernatants and lysates of the oral *Actinomyces* on the proliferation of *C. albicans* that suggested an antagonistic interaction between oral *Actinomyces* and *C. albicans*, and that secreted bacterial factors play important roles in this process. The inhibitory effect was seen with the cell suspensions, culture supernatants and bacterial lysates. The cell suspensions when added together may exert inhibition due to the Jameson effect where high concentrations of the *Actinomyces* could have reached the point at which growth inhibition occurred for both microbes, due to lack of metabolites or other requirements. However, the cell suspensions contained not only live bacterial cells but also secreted metabolites. *Actinomyces* are also able to produce many metabolites with anti-fungal activity, including nikkomycin and geldanamycin. Because there are also polysaccharides and
proteins in the bacterial lysates, these results might further suggest that the source of the inhibitory substances and their inhibitory effects might depend on substances secreted by the living bacteria.

The first step in colony formation and invasion by *C. albicans* is its adhesion to the host epithelial cell, which reflects its strong pathogenicity. Given the result of the adhesion study, we infer that the adhesion of *C. albicans* to BEC was inhibited by some component of the oral *Actinomyces* supernatant, which might combine with adhesion-related polysaccharides and glycolipids or the adhesive receptors of *C. albicans*.

The acid protease and phospholipase activities of *C. albicans* are also critical virulence factors, mainly related to its invasion of the host. *C. albicans* is suited for growth in acidic conditions, in which the secreted proteases have strong hydrolytic activity, whereas the appropriate pH for the growth of *Actinomyces* is about 6.7–7.0. In the acid protease and phospholipase activity experiments, the pH value of the suspensions of experimental groups was about 4.5–5.0 (specific data not shown). Therefore, in our study, the oral *Actinomyces* clearly inhibited the acid protease and phospholipase activities of *C. albicans*, which might be associated with the change of acidic conditions. However, the specific mechanisms require further study.

The growth of germ tubes by *C. albicans* plays an important role in increasing its pathogenicity, leading to infection. Because of the relationship between germ tube formation and the adhesion of *C. albicans*, hyphae formation might be involved in the adhesion mechanism. Hyphae formation also depends on specific environmental factors, so the secretions of oral *Actinomyces* could change the growth environment of *C. albicans*, inhibiting its formation of hyphae. A biofilm is a dense microbial network consisting mainly of thalli, hyphae, pseudohyphae and extracellular matrix that provides the main mode of survival for *C. albicans*. This form has obvious advantages in improving its pathogenicity and capacity for growth. Research has suggested that hyphae formation is closely related to biofilm formation. In other words, it would be hard to form a stable biofilm without hyphae formation. Therefore, we speculate that the inhibition of the *C. albicans* biofilm by oral *Actinomyces* results from a reduction in fungal hyphae.

The results of the experiments with the clinical *C. albicans* strains confirmed that the three oral *Actinomyces* have inhibitory effects of the proliferation, adhesion, metabolic enzymes, hyphae formation and biofilm development of *C. albicans*. This suggests that these inhibitory effects also exist in

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Fig. 2 – Effects of oral *Actinomyces* on biofilm formation by *C. albicans*. (A) and (B), *Candida albicans* biofilm of an experimental group (Av supernatant + CA) was sparse when observed with scanning electron microscopy (SEM) at ×2000 and ×5000. (C) and (D), *Candida albicans* biofilm of the control group was denser than that of the experimental group.
the oral environment. However, the sample of clinical C. albicans strains selected was small and the oral microecological environment is very complex. Moreover, differences might exist between clinical oral Actinomyces strains and the ATCC type strain, so larger sample sizes or an oral candidiasis model is required to verify our conclusions.

5. Conclusion

This preliminary study suggests that oral Actinomyces have inhibitory effects on C. albicans by affecting proliferation, adhesion, metabolic enzyme activity, hyphae formation and biofilm development. Actinomyces may be an important part of the oral flora that is antagonistic to C. albicans and help prevent development of Candida-associated stomatitis.

Conflict of interest

The authors declare that they have no conflict of interest.

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