



ORIGINAL ARTICLE

In vitro antifungal effect and inhibitory activity on biofilm formation of seven commercial mouthwashes

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OBJECTIVE: To investigate the antifungal ability of seven over-the-counter mouthwashes against planktonic and sessile *Candida albicans* and *Candida krusei*.

MATERIALS AND METHODS: The seven mouthwashes studied were Listerine[®], compound chlorhexidine solution, povidone iodine solution (PV-I), cetylpyridinium chloride solution, Colgate[®] Plax, Crest[®] Prohealth Mouthwash, and NaHCO₃. The antifungal ability of each mouthwash against ATCC90028, ATCC6258, and 10 clinical *C. albicans* isolates was tested using disk diffusion tests, the broth microdilution method, and biofilm testing with two different XTT-reduction assays. Fluconazole was used as a positive control, and the experiments were performed in triplicate.

RESULTS: Chlorhexidine and cetylpyridinium chloride had the largest inhibition zones for ATCC90028 and ATCC6258 (18.6 ± 3.5 and 19 ± 1.6 mm, respectively). Cetylpyridinium chloride was the most effective at inhibiting all of the planktonic *C. albicans* strains and ATCC6258 with the minimum inhibitory concentration (MIC). As the maturity of the biofilms increased, the change in sessile cell MIC of the mouthwashes was much smaller than that of fluconazole. For the mature biofilms, chlorhexidine, PV-I, and cetylpyridinium chloride produced the greatest reductions in metabolism (60–80%).

CONCLUSION: Most of these seven mouthwashes had significant antifungal activity for both planktonic and sessile *Candida* species.

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Keywords: mouthwashes; antifungal effect; *Candida*; biofilm

Introduction

Oral candidiasis is a common opportunistic mycosis caused by *Candida* species, most commonly *Candida albicans*. This disease typically affects immunosuppressed patients, such as cancer, transplant, and acquired immunodeficiency syndrome (AIDS) patients, as well as older people wearing dentures. Traditionally, oral candidiasis is classified into acute and chronic forms, and the signs and symptoms are dependent upon the type. Apart from the appearance of the lesions, candidiasis can also cause pain, burning sensation, and altered taste (Prabhu *et al*, 1992).

Several *C. albicans* virulence factors have been proposed. Of these, biofilm formation is considered to be the most important. The biofilms formed by *C. albicans* adhere to the surfaces of the oral mucosa, teeth, and prosthetic appliances. The key characteristic of these biofilms is antifungal resistance, as they can produce a barrier impenetrable to host defenses and antimicrobial therapy (Donlan and Costerton, 2002).

Given the rise in the number of severely immunocompromised patients following the AIDS epidemic, improved life-sustaining therapy, and aggressive anticancer therapy, the incidence of oral candidiasis has increased (Cutler *et al*, 2007). This requires systemic and topical therapeutic approaches. Fluconazole is frequently used to treat candidiasis, especially in patients with underlying systemic infections, but the recurrence of infection and the side effects of fluconazole (FLZ) sometimes restrict its application (Vensel, 2002). In addition, *Candida* spp. other than *C. albicans*, such as *Candida krusei* and *Candida glabrata*, which are resistant toazole antifungals, are also detected frequently in the oral cavity (Bagg *et al*, 2003).

Commercial mouthwashes with antifungal agents have many advantages for use as preventives or adjunctive therapy, such as safety, fewer side effects, and easily obtainable (Gagari and Kabani, 1995). To our knowledge, however, no report has examined the antifungal activity of commercial mouthwashes available in China. Therefore, this *in vitro* study investigated the susceptibility of planktonic and sessile *C. albicans* (ATCC90028 and strains isolated from patients with oral candidiasis) and *C. krusei* to FLZ and a range of over-the-counter mouthwashes.

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Materials and methods

Fungal culture and storage conditions

Twelve *Candida* isolates (11 *C. albicans* and one *C. krusei*) were studied. *Candida albicans* ATCC90028 and *C. krusei* ATCC6258 are the species type strains. The other 10 *C. albicans* strains were clinical isolates from denture stomatitis patients detected at the Peking University School of Stomatology.

The isolates were propagated on Sabouraud agar (Jinzhong Science and Technology Development, Tianjin, China) at 37°C. After 48 h, three to five mature colonies were resuspended in RPMI-1640 buffered with morpholinopropanesulfonic acid [MOPS (Sigma, Dorset, UK)] at a cellular density equivalent to $1-5 \times 10^6$ CFU ml⁻¹.

All working stocks of *Candida* were maintained at 4°C on Sabouraud agar and stored at -80°C for long-term storage.

Antifungal agents and mouthwashes

The antifungal agents used in this study were FLZ (Chinese and Western Three-Dimensional Pharmaceutical, Shanghai, China) and sodium bicarbonate tablets (NaHCO₃; Yanjing Pharmaceutical, Beijing, China, lot no. 111106), which were dissolved in water to make a 5% final concentration solution.

The commercial mouthwashes evaluated along with their active ingredients and recommended application time are as follows: compound chlorhexidine solution (CHL; South China Pharmaceutical, Shenzhen, China, lot no. 20111105) containing chlorhexidine gluconate (0.12% w/v) and metronidazole (0.02% w/v), no recommended application time; povidone iodine solution (PV-I; Yongan Pharmaceutical, Chengdu, China, lot no. 111001) containing PV-I (0.01% w/v), no recommended application time; cetylpyridinium chloride solution (CPC; Hengsheng Pharmaceutical, Nanjing, China, lot no. 111005) containing cetylpyridinium chloride (0.1% w/v), 60-s rinse; Listerine[®] (Johnson & Johnson, Shanghai, China, lot no. 1010145) containing eucalyptol (0.092% v/v), thymol (0.064% v/v), methyl salicylate (0.060% v/v), menthol (0.042% v/v), and ethanol (26.9% v/v), 30-s rinse; Colgate[®] Plax (Colgate Palmolive, Guangzhou, China, lot no. 1123199) containing main effective components cetylpyridinium chloride (0.05% w/v) and fluoride (0.022% w/v), 30-s rinse; and Crest[®] Prohealth mouthwash (P&G, Guangzhou, China, lot no. 0317852511) containing cetylpyridinium chloride (0.05% w/v), 60-s rinse.

Antifungal susceptibility testing

Disk diffusion test. Disk diffusion testing was carried out according to the reference document guidelines (CLSI M44-A, 2002) on Mueller-Hinton agar (90mm diameter; Oxoid, Hampshire, UK) supplemented with 2% dextrose and 0.5 mg l⁻¹ methylene blue. Antifungal disks were prepared by embedding them with the drug. The positive control disks (6mm diameter; Oxoid) were embedded with a final FLZ concentration of 25 µg per disk. For the other mouthwashes, as standard concentrations are not mentioned in CLSI M44-A, we added the maximum loading volume

(13 µl per disk) of the original concentration of the mouthwashes for each blank disk.

A sterile cotton swab was dipped into the fungal suspension, and the excess fluid was removed. The inoculum was spread by smearing the swab evenly over the agar surface, and the plates were dried at 27°C for 15 min before placing the antifungal disks on the surface. Each plate was separated into three equal zones. A disk with mouthwash, a disk with FLZ as a positive control, and a blank disk were applied in the center of each zone. After incubation at 37°C for 48 h, the inhibition diameters around the disks were measured.

Broth microdilution method. Antifungal testing to determine the minimum inhibitory concentration (MIC) of planktonic cells was performed by following the CLSI M-27A broth microdilution method. The fungal suspension was adjusted to $1-5 \times 10^6$ CFU ml⁻¹ and then diluted with RPMI-1640 to a final density of $0.5-2.5 \times 10^3$ CFU ml⁻¹. The inoculum and drugs were added to flat-bottomed 96-well microtiter plates (Corning, Big Flats, NY, USA) with serially twofold diluted concentrations (FLZ, 0.125–64 µg ml⁻¹; NaHCO₃ and PV-I, 1–1:512 clinical concentration dilution; the others, 1:2–1:1024 clinical concentration dilution). Drug-free and fungus-free wells were included as controls. Each condition was repeated three times. Then, the plates were incubated at 37°C. The end points were read visually and measured using the optical density (OD) value after 48 h.

Biofilm antifungal testing. Biofilm antifungal testing was performed using the 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay in two different ways: the antifungal activities of mouthwashes on developing biofilms and a time-kill assay of mature biofilms.

First, a biofilm of *C. albicans* ATCC90028 was formed on flat-bottomed 96-well microtiter plates by adding 100 µl fungal suspension to each well and incubating the plates for 2, 4, 8, 12, and 24 h at 37°C. At each time point, the medium was aspirated and non-adherent cells were removed by softly washing the biofilms three times in sterile phosphate-buffered saline (PBS). Then, the FLZ and mouthwashes were added to the wells in serially twofold diluted concentrations (FLZ 0.5–1024 µg ml⁻¹; mouthwash concentrations as above). Then, the plates were incubated for 48 h at 37°C. Finally, the biofilm metabolic activity was measured using the XTT-reduction assay. Drug- and biofilm-free wells were included to serve as negative and blank controls, respectively.

Second, biofilms of *C. albicans* ATCC90028 and the other 10 clinical strains were formed on flat-bottomed 96-well microtiter plates and incubated for 48 h at 37°C. After washing three times with sterile PBS, the biofilms were immersed in the mouthwashes at the working concentrations at room temperature for 30, 60, and 120 s. Then, the biofilms were washed again with sterile PBS before evaluating the biofilm metabolic activity. Each well had three copies.

The biofilm activity was measured semiquantitatively using an XTT-reduction assay, adapted from previous

reports (Shuford *et al.*, 2007). XTT was prepared as a saturated 0.5 g l⁻¹ solution in PBS and stored at -80°C. XTT (91 µl) and menadione (9 µl; 1 mM prepared in acetone; Sigma) were added to each well. Then, the plates were incubated in the dark for 2 h at 37°C. The absorbance was measured at 492 nm using a microtiter plate reader. The colorimetric reduction in the XTT assay is directly correlated with the metabolic activity of the biofilms.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 (SPSS China, Shanghai, China). For multiple comparisons, the Kruskal–Wallis test and chi-squared statistic were used to determine whether any groups exhibited a significant difference in biofilm metabolism. If the Kruskal–Wallis test demonstrated that at least one group was statistically different, a *post hoc* analysis using the Mann–Whitney *U*-test and Bonferroni correction was used to adjust the significance (*P*-value) for the number of comparisons.

Results

The results of the FLZ quality control groups of *C. albicans* ATCC90028 and *C. krusei* ATCC6258 all conformed to the standard range.

Disk diffusion testing

With disk diffusion testing, for *C. albicans* ATCC90028, CHL was the most effective (inhibition zone diameter 18.6 ± 3.5 mm), followed by CPC, Colgate®, Crest®, PV-I, and Listerine®. For *C. krusei* ATCC6258, CPC was the most effective (19 ± 1.6 mm), followed by Colgate®, Crest®, CHL, Listerine®, and PV-I. NaHCO₃ did not form an inhibition zone for either organism (Figure 1, Table 1).

Broth microdilution method

In the planktonic state, the *C. albicans* oral isolates, ATCC90028, and *C. krusei* ATCC6258 were susceptible to all of the mouthwashes tested. CPC, Colgate®, and Crest®, for which the main active ingredient is cetylpyridinium chloride, best inhibited the *C. albicans* strains and

Table 1 The inhibition zone diameters of different mouthwashes for inhibition of *Candida albicans* and *Candida krusei* (mm)

| Mouthwash | <i>Candida albicans</i> ATCC90028 | | <i>Candida krusei</i> ATCC6258 | |
|--------------------|--------------------------------------|-----------|--------------------------------|-----------|
| | Mean ± s.d. | 95% CI | Mean ± s.d. | 95% CI |
| FLZ | 30.1 ± 3.1 | 29.3–30.8 | 7.9 ± 0.8 | 7.3–8.5 |
| CHL | 18.6 ± 3.5 | 16.1–21.1 | 14.8 ± 0.8 | 13.8–15.8 |
| PV-I | 9.1 ± 1.4 | 8.4–9.9 | 7.4 ± 0.6 | 6.7–8.1 |
| CPC | 14.2 ± 2.7 | 12.7–15.7 | 19 ± 1.6 | 17.3–20.1 |
| Listerine | 8.0 ± 3.4 | 5.9–10.2 | 8.2 ± 0.6 | 7.5–8.9 |
| Colgate | 13.4 ± 3.5 | 11.4–15.3 | 18.2 ± 1.1 | 16.8–19.6 |
| Crest | 10.8 ± 2.5 | 9.4–12.2 | 16 ± 1.0 | 14.8–17.2 |
| NaHCO ₃ | – | – | – | – |

FLZ, fluconazole; CHL, compound chlorhexidine solution; PV-I, povidone iodine solution; CPC, cetylpyridinium chloride solution.

C. krusei. CHL also gave a reasonable result for the *C. albicans* strains (MIC₅₀ 1.25% and MIC₉₀ 1.56% of the commercially available concentration) and *C. krusei* (MIC₅₀ and MIC₉₀ 0.78%). NaHCO₃ showed a certain degree of antifungal effect with an MIC₅₀ of 80% for the *C. albicans* strains and 100% for *C. krusei* (Table 2).

Biofilm antifungal testing

The first method demonstrated that as the length of biofilm growth increased, the sessile minimum inhibitory concentration (SMIC) also increased for all drugs. Compared with FLZ (1–1024 µg ml⁻¹), the changes for the mouthwashes were smaller (CHL 0.39–0.78% of the commercial concentration, PV-I 25–50%, CPC 0.2–0.39%, Listerine® 25–50%, Colgate® 0.39–0.78%, Crest® 0.39–0.78%, and NaHCO₃ 50–100%). After 24 h, the biological membrane had matured, and the SMIC of FLZ increased to 1024 µg ml⁻¹, while the SMICs of the mouthwashes were still lower than the clinical concentration (Figure 2, Table 3).

In the time-kill assay of the metabolism of mature biofilms, the mature biofilms were exposed to mouthwashes at clinical concentrations for 30, 60, and 120 s.

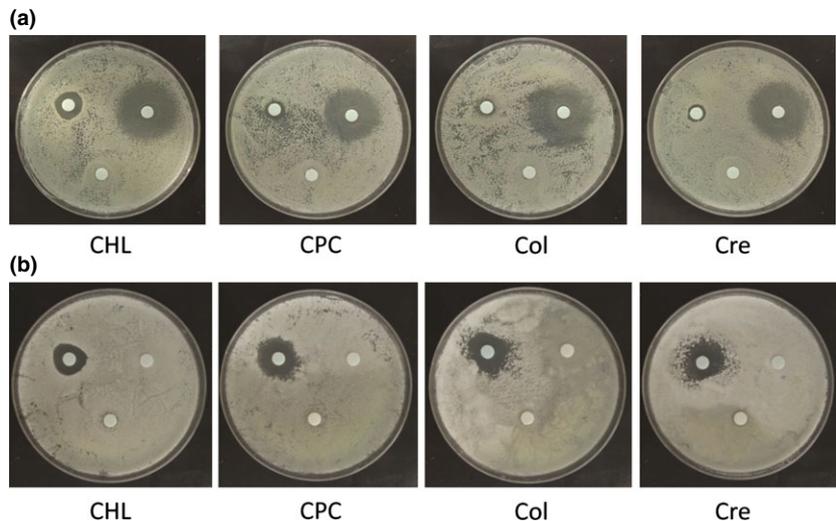


Figure 1 (a) The disk diffusion testing results for *Candida albicans* ATCC90028. For each agar plate, the upper-left disk was impregnated with mouthwash, the upper-right with fluconazole (FLZ), and the bottom disk was a blank. (b) The disk diffusion testing results for *Candida krusei* ATCC6258. For each agar plate, the upper-left disk was impregnated with mouthwash, the upper-right with FLZ, and the bottom disk was a blank.

Table 2 Antifungal susceptibility profiles of planktonic *Candida albicans* and *Candida krusei*

| Mouthwash | <i>C. albicans</i> (<i>C. albicans</i> ATCC90028 and 10 clinical isolates) | | | <i>C. krusei</i> ATCC6258 | |
|-----------------------------------|---|-------------------|-------------------|---------------------------|-------------------|
| | MIC ₅₀ range | MIC ₅₀ | MIC ₉₀ | MIC ₅₀ | MIC ₉₀ |
| Fluconazole (FLZ) ^a | 0.25–2 | 0.60 | – | 32 | – |
| Compound chlorhexidine solution | 0.78–1.56 | 1.25 | 1.56 | 0.78 | 0.78 |
| Povidone iodine solution | 12.5–25 | 16.25 | – | 25.00 | – |
| Cetylpyridinium chloride solution | 0.05–0.39 | 0.20 | 0.27 | 0.05 | 0.05 |
| Listerine | 25–50 | 44.44 | – | 6.25 | 12.50 |
| Colgate | 0.1–1.56 | 0.58 | 0.67 | 0.05 | 0.10 |
| Crest | 0.2–1.56 | 0.53 | 0.72 | 0.10 | 0.10 |
| NaHCO ₃ | 50–100 | 80 | – | 100 | – |

Each concentration described represents a proportion of the commercially available concentration.

^aUnit of FLZ: $\mu\text{g ml}^{-1}$.

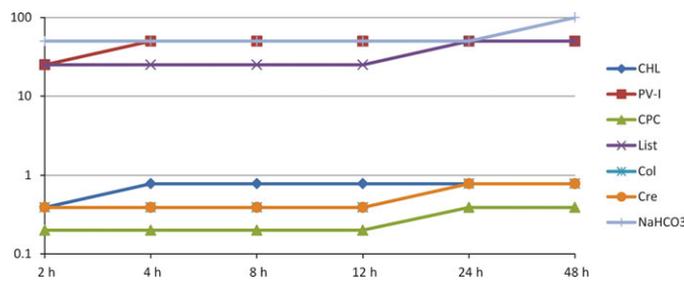


Figure 2 The antifungal activities of the mouthwashes on developing biofilms. As the biofilms matured, the SMIC₅₀ of each mouthwash increased. In this figure, the polylines of Col and Cre were completely overlapped and the polyline of CHL was overlapped with them on points of 24h and 48h. The polylines of PV-I and NaHCO₃ were also overlapped on points of 4h, 8h, 12h and 24h. And the polylines of PV-I and List were overlapped on points of 24h and 48h

CHL, PV-I, and CPC were the most effective agents, producing 60–80% reductions in metabolism (Figure 2). No significant differences among these three agents were observed. Crest[®], Listerine[®], and Colgate[®] induced 20–30% reductions in metabolism, with Crest[®] more effective than the others. CHL, PV-I, and CPC were all significantly superior to Crest[®], Listerine[®], and Colgate[®] ($P < 0.05$). NaHCO₃, which resulted in only a 5–8% metabolic reduction, was significantly inferior to the others ($P < 0.05$). For CHL, the 120-s treatment period was significantly superior to the 30-s treatment ($P < 0.05$). For

PV-I and NaHCO₃, the 60- and 120-s treatments were superior to the 30-s treatment (Figure 3).

Discussion

Oral candidiasis is a common opportunistic mycosis, especially in immunosuppressed patients. *Candida albicans* continues to be the predominant organism associated with oral yeast infections (Coco et al, 2008; Pereira-Cenci et al, 2008). Given the increasing incidence of candidiasis since 1970, many systemic and topical therapeutic approaches are required. It has been suggested that prolonged or repeated exposure to FLZ is associated with the emergence of FLZ resistance among strains of *C. albicans* (Johnson et al, 1995; Lopez et al, 2001).

In addition, the ability of *C. albicans* to populate a surface and produce a biofilm provides protection against mechanical force and antifungal agents. Therefore, *C. albicans* biofilms are much more resistant to a range of antifungal drugs than are free-living planktonic cells. In this study, we report that FLZ, a conventional antifungal agent, although effective against planktonic cells, shows reduced activity against *C. albicans* biofilms *in vitro*. However, four of the mouthwashes (CPC, CHL, Colgate[®], and Crest[®]) studied exhibited significant anti-biofilm activity *in vitro*, suggesting that they are alternative therapeutic strategies for oral candidiasis.

Our results demonstrated that all seven of the mouthwashes tested inhibited the growth of *C. albicans* on

Table 3 SMIC₅₀ for *Candida albicans* ATCC90028 for biofilms matured for different durations (%)

| Mouthwash | 2 h | 4 h | 8 h | 12 h | 24 h |
|-----------------------------------|------|------|------|------|------|
| Fluconazole (FLZ) ^a | 1 | 1 | 2 | 8 | 1024 |
| Compound chlorhexidine solution | 0.39 | 0.78 | 0.78 | 0.78 | 0.78 |
| Povidone iodine solution | 25 | 50 | 50 | 50 | 50 |
| Cetylpyridinium chloride solution | 0.2 | 0.2 | 0.2 | 0.2 | 0.39 |
| Listerine | 25 | 25 | 25 | 25 | 50 |
| Colgate | 0.39 | 0.39 | 0.39 | 0.39 | 0.78 |
| Crest | 0.39 | 0.39 | 0.39 | 0.39 | 0.78 |
| NaHCO ₃ | 50 | 50 | 50 | 50 | – |

Each concentration described represents a proportion of the commercially available concentration.

^aUnit of FLZ: $\mu\text{g ml}^{-1}$.

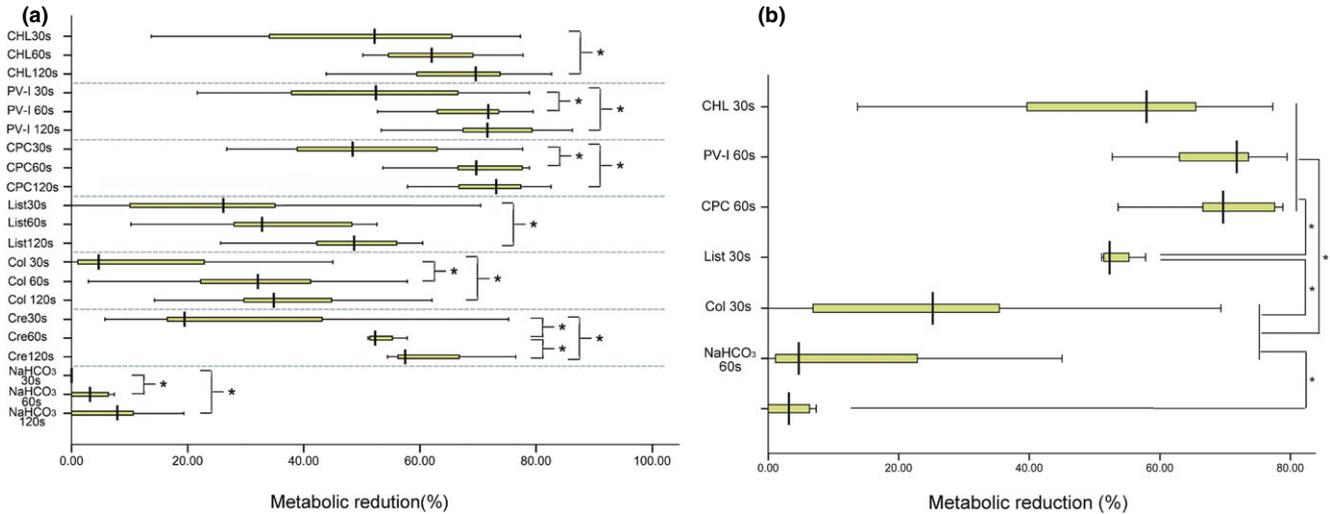


Figure 3 (a) Boxplots depicting the time-kill assay of the metabolism of a mature biofilm of *Candida albicans* ATCC90028. Each box indicates the lower and upper quartiles. The central line is the median. The whiskers indicate 10% and 90% (**P* < 0.05). (b) Boxplots illustrating the results of management for the time indicated by the manufacturers or our previous test (**P* < 0.05)

Sabouraud agar. Moreover, the mouthwashes, especially CHL, CPC, Colgate®, and Crest®, were very active against planktonic cells in broth in the microdilution method. These four mouthwashes each achieved a very low MIC₉₀. These results indicate that the mouthwashes are effective at killing the planktonic cells in saliva and preventing biofilm formation.

In the biofilm antifungal assay, after 24-h culture, all of the mouthwashes except NaHCO₃ had a low SMIC₅₀, while the biofilms showed FLZ resistance. The effective SMIC₅₀ values of CHL, CPC, Colgate®, and Crest® were equal to the breakpoint for planktonic MIC testing and were lower compared with the other mouthwashes. These results are consistent with the reports that chlorhexidine (0.12%) and Listerine® mouthwashes were effective against fungal biofilms (Meiller *et al*, 2001; Lamfon *et al*, 2004).

Uppuluri *et al* (2010) reported a significant correlation between *C. albicans* biofilm formation and mortality due to *candida*, whereas mortality was not associated with the antifungal sensitivity of planktonic cells. In our study, the antifungal sensitivity of *C. albicans* decreased as the biofilm matured over 24 h, which indicated that *C. albicans* does not require the acquisition of defined molecular mechanisms to resist treatment. Instead, the ability to form biofilms on various substrates provides adequate protection from antifungal agents.

Moreover, we found that the capacity to reduce the biofilm metabolism of the mouthwashes increased with the duration of exposure. Most of the mouthwashes were significantly more effective at 60- vs 30-s exposure times, while the differences between the 60- and 120-s exposures were not significant. These results are consistent with the manufacturer’s instructions, which recommend the mouthwashes be used for 1 min.

There are some drawbacks to the use of over-the-counter mouthwashes, including superficial staining of enamel, burning sensation, and altered taste. Many also have high alcohol contents, which has been implicated in oral cancer

(Conway, 2009; La Vecchia, 2009). Moreover, as our data were generated entirely *in vitro*, appropriate clinical trials are needed to establish both the effectiveness and patient acceptability of these over-the-counter preparations *in vivo*.

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Author contributions

J. Fu and P. Wei carried out the study and wrote the manuscript. C. Zhao and C. He helped to collect clinical isolates. H. Hua and Z. Yan designed the study and revised the manuscript.

Conflict of interest

We declared we have no conflict of interest with any of the companies which manufacture the mouthwashes involved in this study.

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