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Voriconazole inhibits cross-kingdom interactions between *Candida albicans* and *Actinomyces viscosus* through the ergosterol pathway

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ABSTRACT

Candida albicans and *Actinomyces viscosus* are prominent microbes associated with dental root caries. The aim of this study was to investigate the effect of *C. albicans* on *A. viscosus* biofilms and to identify the mechanisms associated with this interaction. *A. viscosus* and *C. albicans* strains (wide-type and mutants) were used to form biofilms *in vitro* and *in vivo*, which were subsequently analysed by crystal violet assay and scanning electron microscopy (SEM) to investigate the effect of *C. albicans* on *A. viscosus* growth. A viable plate count and survival curve for *C. albicans* mutants and *A. viscosus* combinations were used to identify which *C. albicans* pathway was crucial for cross-kingdom interactions. Voriconazole was used to block their interactions both *in vitro* and *in vivo*. SEM, fluorescence in situ hybridisation (FISH), quantitative PCR and survival curve analyses were performed to evaluate the activity of voriconazole on *C. albicans* and *A. viscosus* interactions. The biomass and virulence of mixed-species biofilms were significantly enhanced compared with the *A. viscosus* biofilm alone. However, this was not observed in the mixed-species biofilms with the *C. albicans* mutant *erg11*Δ/Δ *in vitro* and *in vivo*, indicating that azoles may work on the mixed-species biofilms. As expected, voriconazole can effectively reduce the biomass of mixed-species biofilms. A high concentration of voriconazole (1 μg/mL) reduced the abundance of *C. albicans*, whilst a low voriconazole concentration (0.25 μg/mL) blocked their interactions similar to the effect of the *erg11*Δ/Δ mutant. Voriconazole may be a candidate strategy to combat root caries pathogens.

1. Introduction

Dental root caries is a biofilm-dependent, chronic infectious disease that occurs on the surface of the cementum, a substance covering the root of a tooth. It is a major cause of tooth loss in older adults [1]. Many microbial species are identified to be associated with root caries. *Actinomyces viscosus* can be isolated from almost all root caries. The abundance of *A. viscosus* can reach 45% of the total bacterial biomass from root caries lesions, indicating a strong relationship between *A. viscosus* and root caries [2,3]. *Candida albicans* is the most common fungus in the human oral cavity that can form multispecies biofilms and as a consequence promote the development of various polymicrobial diseases, including dental caries [4]. *C. albicans* is frequently detected along with *A. viscosus* in plaque from root caries [5] and can also be aggregated with *A. viscosus in vitro*. The surface proteins and carbohydrates of *C. albicans* and *A. viscosus* may be related to coaggregation [6]. However, how their interactions contribute to root caries is not clear.

Antifungal azole drugs have been shown to be suitable for the adjunctive treatment of dental caries, indicating the important role of fungi in caries and that inhibition of fungi benefits dental caries treatment [7]. Voriconazole is a second-generation, broad-spectrum triazole antifungal drug. It inhibits the fungal enzyme lanosterol 14α-demethylase and block thus ergosterol biosynthesis. Voriconazole is also capable of inhibiting the transformation of *C. albicans* from spores to invasive hyphae [8]. However, to our knowledge, the activity of voriconazole on cross-kingdom biofilms has not been reported.

The Greater wax moth (*Galleria mellonella*) has an innate immune response system and a short life cycle, making it an easy-to-use *in vivo* model to evaluate the virulence of interactions between bacteria and fungi, especially for screening different mutant strains [9,10]. Results from *G. mellonella* models are highly consis-

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tent with those from mice and humans. A benefit of the *G. mellonella* model is a reduction in the number of experimental mammals used, especially for initial screening and analysis [11–16]. In the current study, interactions between *C. albicans* (wild-type and mutants) and *A. viscosus* were analysed *in vitro* and *in vivo*. The activity of voriconazole to block their cross-kingdom interactions *in vitro* and *in vivo* was also evaluated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Candida albicans strains used in this study are listed in Supplementary Table S1. *Actinomyces viscosus* ATCC 19246 was grown anaerobically (90% N_2, 5% H_2 and 5% CO_2) at 37°C in BHY medium (brain–heart infusion medium containing 5 g/L yeast extract) [17]. *Candida albicans* strains were grown aerobically at 37°C in YNB (yeast–nitrogen–base) medium (Difco Laboratories, Detroit, MI) [18]. Combinations of *A. viscosus* and *C. albicans* were grown in YNBB medium containing 6.7 g/L YNB (Difco Laboratories), 75 mM Na_2HPO_4–NaH_2PO_4 (pH 7.3), 2.5 mM *N*-acetylglucosamine (Sigma-Aldrich, Taufkirchen, Germany), 2 g/L casamino acids (Becton Dickinson & Co., Sparks, MD) and 5 g/L sucrose [18].

2.2. Biofilm formation and treatment

Pre-cultures of *A. viscosus* and *C. albicans* strains were inoculated from single colonies grown for 24 h and harvested by centrifugation (4000 rpm, 10 min, 4°C). The suspension of each microorganism was standardised in YNBB medium to an optical density at 600 nm (OD_{600}) of 0.1. Single and mixed biofilms were produced on Corning® Costar-® 96-well or 24-well plates (Corning Inc., Corning, NY). For single-species and dual-species inocula, equal volumes of each strain were inoculated into the plates, respectively (200 µL final volume for 96-well plates and 1 mL final volume for 24-well plates) [18]. Biofilms were incubated anaerobically for 24 h at 37°C. Voriconazole was then added to the biofilms at different concentrations and the biofilms were incubated anaerobically for 12 h at 37°C [19].

2.3. Effect of wild-type Candida albicans on the biomass of Actinomyces viscosus analysed by crystal violet (CV) assay

Biofilms were formed in 96-well plates as described above and the biomass was quantified by a CV assay according to Alnuaimi et al [20]. Initially, the biofilm in each well of the 96-well plates was washed with sterilised phosphate-buffered saline (PBS) and then 200 µL of anhydrous ethanol was added for 30 min to fix the biofilms. The supernatant was then removed and the plates were air-dried for 30 min. Subsequently, 200 µL of a 0.1% CV solution was added to the wells for 20 min and the plate was then washed twice with PBS to remove excess CV. Then, 200 µL of 33% acetic acid was added to release CV, and 200 µL of the solution was transferred to another 96-well plate to measure the absorbance at 600 nm using a microtitre plate reader (Bio-Rad, Hercules, CA).

2.4. Effects of wild-type Candida albicans on the biofilm structure of Actinomyces viscosus analysed by scanning electron microscopy (SEM)

Biofilms were produced on 24-well plates with a sterilised glass slide. After allowing biofilms to form as described above, the specimens were rinsed three times with PBS and were then fixed with 2.5% glutaraldehyde for 3 h at room temperature. The specimens were then washed with PBS and were dehydrated in a series of aqueous ethanol solutions (30%, 50%, 70% and 90%) for 30 min each time, followed by immersion in 100% alcohol for 1 h. Samples were then subjected to drying with CO_2 and were sputter-coated with gold/palladium. Analysis was carried out using a Quanta 200 scanning electron microscope (FEI, Hillsboro, OR) [21].

2.5. Effect of Candida albicans mutants on the proliferation of Actinomyces viscosus analysed by the viable plate count method

Suspensions of *A. viscosus* and *C. albicans* wild-type and the *phr1*Δ/Δ, *phr2*Δ/Δ, *erg3*Δ/Δ and *erg11*Δ/Δ mutants were inoculated into 24-well plates for dual-species inocula. The plates were then cultured continuously for 24 h and the number of CFU was calculated. Samples were ultrasonicated three times at a power setting of 8 W for 10 s each time. Then, 10-fold serial dilutions of the suspension were plated on YNBB agar for total colony counts [22].

2.6. Effect of a Candida albicans mutant on the biofilm virulence of Actinomyces viscosus analysed using Galleria mellonella survival curves

Galleria mellonella (KaiDeRuiXin Co. Ltd., Tianjin, China) in the final stage of the larval phase were stored in the dark and were used within 7 days. Nine randomly chosen *G. mellonella* larvae with similar weights and sizes (300–350 mg) were used per experimental group. Larvae were kept without food throughout the experiment. The bodies of larvae were rinsed with 75% alcohol before the injection treatments. Standardised suspensions of *C. albicans* wild-type or the *erg11* mutant (10^4 cells/larva, sublethal concentration for *G. mellonella*) were inoculated into the haemolymph of larva using a Hamilton syringe (Hamilton Inc., Reno, NV) in the left last proleg. Next, standardised suspensions of *A. viscosus* (10^4 cells/larva, sublethal concentration for *G. mellonella*) were inoculated into the haemolymph of larva using a Hamilton syringe via the right last proleg [23,24]. There were two negative control groups in all experiments: (i) a group of larvae mock-inoculated with 10 µL of PBS; and (ii) another group that did not receive any injection to monitor the health status of the *G. mellonella* larvae. Following microbial injections, the *G. mellonella* were incubated at 37°C in a plastic container and were observed after infection. The larvae were considered dead by the absence of movement in response to touch. Experiments with more than two dead caterpillars in either control group were discarded and repeated [25].

2.7. Antifungal drug testing and minimum inhibitory concentration (MIC) determination

Voriconazole (Sigma-Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 160 µg/mL stock concentration and was stored at 4°C until needed [19]. According to a 50% inhibition of both pre-formed biofilm and biofilm formation, cell viability was defined as described by Pierce et al [26]. The MIC_{50} values of voriconazole on the growth of *C. albicans* and *A. viscosus* are shown in Supplementary Table S2. Voriconazole concentrations of 0.125, 0.25, 0.5 and 1 µg/mL were chosen for further studies. Voriconazole was diluted to the assayed concentrations in YNBB medium.

2.8. Effect of voriconazole on biofilm formation of Actinomyces viscosus analysed by an XTT reduction assay

Plates were incubated anaerobically (90% N_2, 5% H_2 and 5% CO_2) at 37°C for 24 h to allow biofilm formation. YNBB medium was then removed and the specimens were gently washed with 200 µL of PBS. Then, 200 µL of voriconazole diluted in YNBB culture medium was inoculated into the wells and the plates were incubated anaerobically at 37°C for 12 h [27]. Total biomass was

quantified by an XTT reduction assay as previously described by Seneviratne et al [28]. Each well was washed with 40 µL of XTT (tetrazolium salt 2,3-bis; Sigma), 2 µL of menadione (Sigma) and 158 µL of PBS and the plate was then incubated at 37°C in the dark for 3 h. The absorbance at 490 nm was then recorded using a microtitre plate reader.

2.9. Effect of voriconazole on biofilm formation of Actinomyces viscosus analysed by scanning electron microscopy

Following formation of biofilm, specimens were rinsed three times with PBS and were then fixed with 2.5% glutaraldehyde for 3 h at room temperature. Specimens were then washed with PBS and were dehydrated in a series of aqueous ethanol solutions (30%, 50%, 70% and 90%) for 30 min each time, followed by 100% alcohol for 1 h. After drying and spraying, samples were observed by SEM [21].

2.10. Effect of voriconazole on Actinomyces viscosus biofilm formation analysed by fluorescence in situ hybridization (FISH)

Biofilm specimens were fixed in 4% paraformaldehyde for 3 h at 4°C and were then rinsed with PBS. FISH was performed as described by Dige et al [29]. Briefly, specimens were subsequently stored in a mixture of ethyl alcohol and PBS (w/w 1:1) at 4°C overnight. Biofilms were permeabilised by exposure to 30 µL of lysozyme and snail enzyme [Sigma-Aldrich; 70 000 U/mL in Tris–HCl (pH 7.5)] for 10 min at 37°C and were rinsed with deionised water. The biofilms were dehydrated in a series of ethanol solutions (50%, 80% and 90%, 3 min) and were dried for 10 min. Biofilms were exposed to 10 µL of hybridisation buffer [0.9 M NaCl, 20 mM Tris–HCl, 0.01% sodium dodecyl sulfate (SDS), 35% formamide, 100 ng of the designated oligonucleotide probe (pH 7.5)] and were incubated at 46°C for 1.5 h in a dark and humid atmosphere. Following hybridisation, specimens were rinsed with washing buffer for 30 min at 48°C and were then washed with deionised water. The oligonucleotide probes EUK516 (5′-ACCAGACTTGCCCTCC-3′) labelled with Cy3 to identify *C. albicans* and ACT218 (5′-CGAGCCCCATCCCCCACCA-3′) labelled with fluorescein isothiocyanate (FITC) to identify *A. viscosus* were used. Probes were synthesised by Sangon Biotech (Shanghai, China). A Leica TCS SP8 inverted confocal laser scanning microscope (Leica Corp., Frankfurt, Germany) was used to observe each sample.

2.11. Effect of voriconazole on biofilm formation of Actinomyces viscosus analysed by quantitative PCR (qPCR)

Total RNA of *C. albicans* was extracted using a FastPure™ RNA Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) [30]. RNA concentrations and purity were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was assessed by agarose gel electrophoresis. The RNA concentration of each sample was adjusted to 200 ng/μL with RNase-free water according to the previously measured RNA concentration. An equal amount of RNA was subjected to cDNA synthesis using a PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology Co. Ltd.). A reverse transcription (RT) reaction mixture (20 µL) containing 5 µL of total RNA, 1 µL of Oligo dT primer (50 µM), 1 µL of PrimeScript™ RT Enzyme Mix I, 4 µL of 5 × PrimeScript™, 1 µL of random 6-mers (100 µM) and 8 µL of RNase-free dH_2O was incubated at 37°C for 15 min, 85°C for 5 s and 4°C termination. cDNA samples were stored at −20°C until use. Forward and reverse primer sequences were designed using Vector NTI software and were synthesised by Sangon Biotech (Supplementary Table S3). The primer concentration was 1 µM. RT-qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR® Premix Ex Taq™ Reagent Kit (TaKaRa Biotechnology Co. Ltd.). All PCR reaction mixtures (20 µL) contained 10 µL of SYBR® Premix Ex *Taq* (2 ×), 2 µL of first strand cDNA, 0.4 µL of forward primer, 0.4 µL of reverse primer, 0.4 µL of ROX Reference Dye (50 ×) and 6.8 µL of RNase-free dH_2O. The programme for amplification was 95°C for 30 s as an initial denaturation step, followed by 40 cycles of PCR consisting of 95°C for 5 s and 60°C for 30 s. 18S rRNA was used as an endogenous reference control for normalisation of the relative expression and the result was analysed using the $2^{-\Delta\Delta CT}$ method [31].

2.12. Effect of voriconazole on biofilm-associated virulence of Actinomyces viscosus analysed using Galleria mellonella survival curves

The effect of voriconazole on the biofilm-associated virulence of *A. viscosus* was evaluated using *G. mellonella* survival curves. Standardised suspensions of *A. viscosus* (10^4 cells/larva) and *C. albicans* (10^4 cells/larva) were inoculated into the haemolymph of larva using a Hamilton syringe in the last left proleg. Then, 0.125, 0.25, 0.5 and 1 µg/mL solutions of voriconazole were inoculated into the haemolymph of larva using a Hamilton syringe in the last right proleg [23,24]. A control group of *G. mellonella* larvae were inoculated with the *erg11*Δ/Δ mutant and *A. viscosus* through the last left and right proleg.

2.13. Statistical analysis

Tukey's post-hoc multiple comparison test and one-way analysis of variance (ANOVA) were performed to compare multiple mean values. Survival curves were analysed by the Kaplan–Meier method and the log-rank test to calculate differences in survival. The level of statistical significance was $P < 0.05$.

3. Results

3.1. Candida albicans promotes Actinomyces viscosus biofilm formation in vitro

The biomass formed by *A. viscosus* and *C. albicans* wild-type combination exhibited a significant increase compared with the biomass formed by single-species biofilms (Fig. 1A). Biofilms formed *in vitro* were then observed by SEM. In the dual-species biofilms, *A. viscosus* represented more biomass, whilst *C. albicans* wild-type was in the yeast form. In the single-species biofilms, less *A. viscosus* cells were observed and *C. albicans* wild-type appeared both a yeast and hyphal forms. The mesh structure between cells in the single-species biofilms was sparse, and less surface matrix was observed compared with the dual-species biofilm (Fig. 1B–D).

3.2. Ergosterol biosynthesis in Candida albicans is critical for cross-kingdom interaction

We then invested which pathways of *C. albicans* contributed to the cross-kingdom interactions. *C. albicans PHR1* and *PHR2* (key genes responsible for pH regulation) null mutants together with *ERG11* and *ERG3* (key genes in the ergosterol biosynthesis pathway) null mutants were co-cultured with *A. viscosus*. In the viable plate count assay, CFU formed by the *C. albicans* wild-type, *phr1*Δ/Δ, *phr2*Δ/Δ and *erg3*Δ/Δ *C. albicans* strains and *A. viscosus* in dual-species biofilms exhibited a significant increase compared with those formed only by the single species of *A. viscosus* (Fig. 2). In contrast, the number of CFU in the *C. albicans erg11*Δ/Δ and *A. viscosus* co-cultured group was significantly reduced, indicating the critical role of the *ERG11* gene in the dual-species interactions (Fig. 2). The *G. mellonell*a model was employed to further

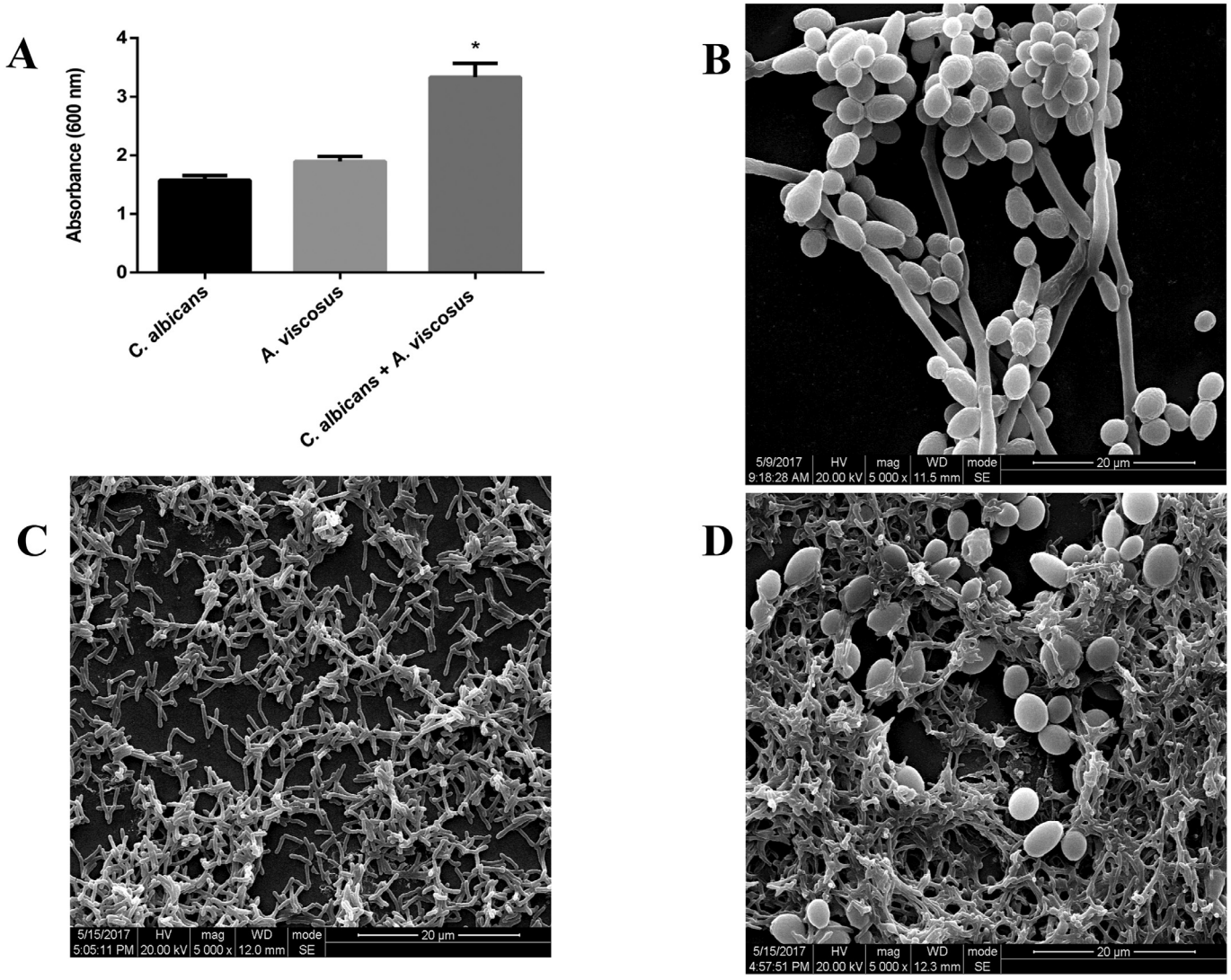

Fig. 1. Quantitative analysis of *in vitro* biofilm formation by crystal violet staining. (A) Biofilm mass was determined and the results demonstrated that mixed biofilms of *C. albicans* wild-type and *A. viscosus* exhibited a significant increase in biomass compared with those formed by single-species biofilms. * $P < 0.05$. (B–D) Scanning electron microscopy of biofilms formed *in vitro* at 24 h by *C. albicans* wild-type (B), *A. viscosus* (C) and *C. albicans* wild-type + *A. viscosus* (D). Original magnification, 5000 ×.

confirm their interactions on virulence *in vivo*. The interaction between *C. albicans* wild-type and *A. viscosus* cells significantly decreased the survival rate of the insects, as approximately 100% of the *G. mellonella* died within 24 h. In contrast, the survival rate of *G. mellonella* infected with *erg11*Δ/Δ and *A. viscosus* combination was significantly enhanced compared with the *C. albicans* wild-type and *A. viscosus* combination, but was similar to the *A. viscosus* single-species infection (Fig. 3).

3.3. Voriconazole blocks the cross-kingdom interaction by regulating the ergosterol pathway

The critical role of the *ERG11* gene for interactions between *C. albicans* and *A. viscosus in vitro* and *in vivo* was highlighted using azole drugs. The voriconazole MIC against *C. albicans* was 0.25 µg/mL, whilst it had no activity against *A. viscosus* (Supplementary Table S2). In the presence of voriconazole, the biomass of the dual-species biofilms containing *C. albicans* wild-type and *A. viscosus* was significantly reduced. As the drug concentration increased, the amount of mixed biofilm decreased gradually. The 0.25 µg/mL and 0.5 µg/mL voriconazole-treated groups showed less biomass than the control group ($P < 0.05$). There was no significant difference between the 0.125 µg/mL voriconazole-treated group and the control group ($P > 0.05$) (Fig. 4).

Voriconazole-treated dual-species biofilms were then observed by SEM. *A. viscosus* exhibited a dense and tight structure in the presence of *C. albicans* wild-type (Supplementary Fig. S1A), which was similar in the 0.125 µg/mL voriconazole-treated group (Supplementary Fig. S1B). However, when the concentration of voriconazole was increased, the biomass in the co-cultures gradually decreased (Supplementary Fig. S1C,D). At a concentration of 1 µg/mL, only a few micro-organisms were observed (Supplementary Fig. S1E). Similar to the voriconazole-treated group, the *C. albicans erg11*Δ/Δ and *A. viscosus* combination group formed very thin biofilms and only a little biomass was observed (Supplementary Fig. S1F).

Voriconazole-treated biofilms were also analysed by confocal laser scanning microscopy. Mixed biofilms of *C. albicans* wild-type and *A. viscosus* were much denser (Fig. 5A) and the biofilm density was decreased in the presence of voriconazole (Fig. 5B,C). When the voriconazole concentration was increased, the degree of microbial coverage became thinner and a notable decrease in

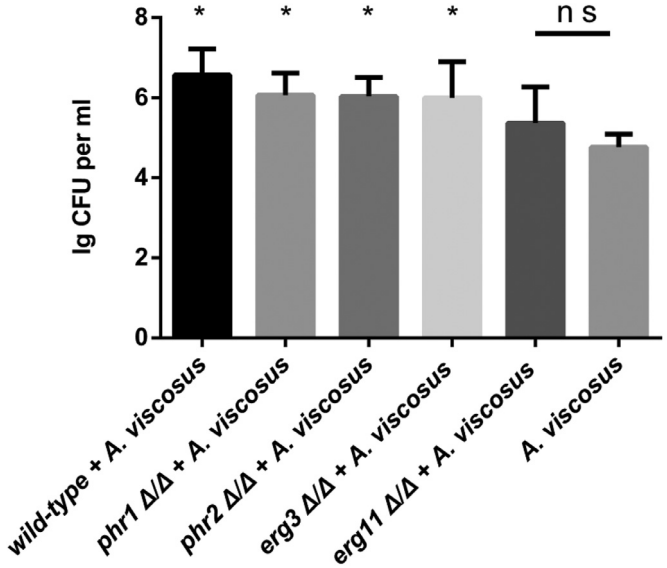

Fig. 2. Quantitative analysis of the effects of *C. albicans* strains (wild-type and mutants) on the proliferation of *A. viscosus* by CFU count. CFU counts did not differ between the *C. albicans erg11*Δ/Δ and *A. viscosus* dual-species and the *A. viscosus* single-species. * $P < 0.05$; ns, not significant.

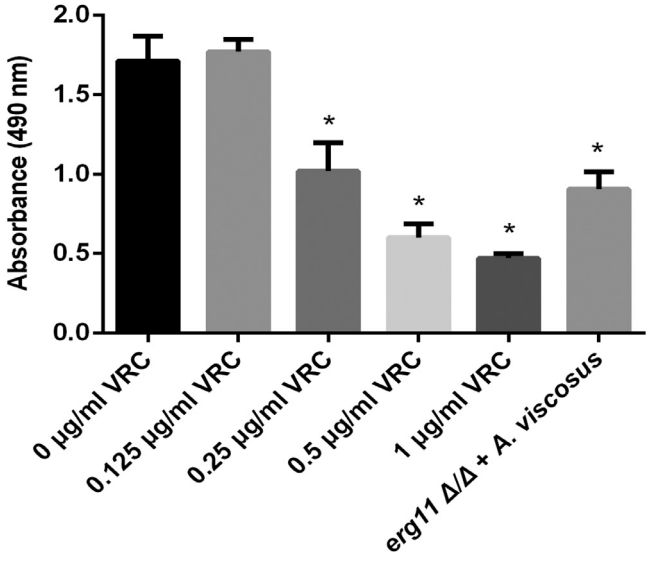

Fig. 4. Biofilm mass determined by XTT reduction assay. Data are the mean ± standard deviation from three independent experiments. Based on absorbance at 490 nm, the results demonstrated that the biomass of the 0.125 μg/mL voriconazole (VRC)-treated group was significantly higher than that of the 1 μg/mL VRC-treated group. There was no statistical difference between the 0.125 μg/mL VRC-treated group and the control group. * $P < 0.05$.

biomass was observed for mixed biofilms treated with 0.5 μg/mL and 1 μg/mL voriconazole (Fig. 5D,E). The biofilm of the *erg11*Δ/Δ and *A. viscosus* combination group was also loose (Fig. 5F). Quantitative analysis of the fluorescence intensity of *A. viscosus* indicated that the presence of a high concentration of voriconazole resulted in a low number of *A. viscosus*. Although there was no significant statistical difference between the 0.125 μg/mL voriconazole-treated group and the control group ($P > 0.05$), *A. viscosus* in the 0.25 μg/mL and 0.5 μg/mL voriconazole-treated groups was less than in the control group ($P < 0.05$; Fig. 5G).

The effect of voriconazole on key genes from the ergosterol biosynthesis pathway was then confirmed. *ERG11* and *ERG3* gene expression in dual-species biofilms containing *C. albicans* wild-type and *A. viscosus* was significantly decreased by voriconazole in a dose-dependent manner ($P < 0.05$), indicating the ability of voriconazole to block the ergosterol biosynthesis of *C. albicans* and thus inhibit the cross-kingdom interactions between *C. albicans* and *A. viscosus* (Fig. 6).

3.4. *Voriconazole treatment of the dual-species infections* in vivo

The effect of voriconazole on the *C. albicans* and *A. viscosus* combination *in vivo* was then tested. In the presence of voriconazole, the survival rate of *G. mellonella* was significantly increased. The survival rate in the 0.5 μg/mL and 1 μg/mL voriconazole-treated group was higher than in the 0.125 μg/mL voriconazole-treated group. No significant difference between the 0.25 μg/mL voriconazole-treated group and the *erg11*Δ/Δ group was observed (Fig. 7).

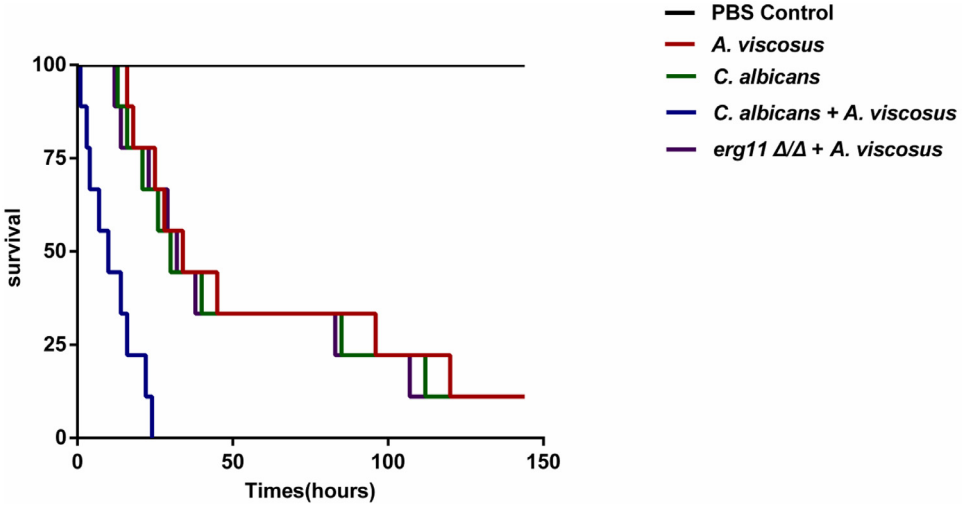

Fig. 3. Survival curves of *Galleria mellonella* larvae in groups inoculated with *A. viscosus*, *C. albicans* wild-type, *C. albicans* wild-type + *A. viscosus*, and *C. albicans erg11*Δ/Δ + *A. viscosus*, as well as the phosphate-buffered saline (PBS) control group. Significant differences were observed between the *C. albicans* wild-type + *A. viscosus* group and the other experimental groups. $P < 0.05$, log-rank test.

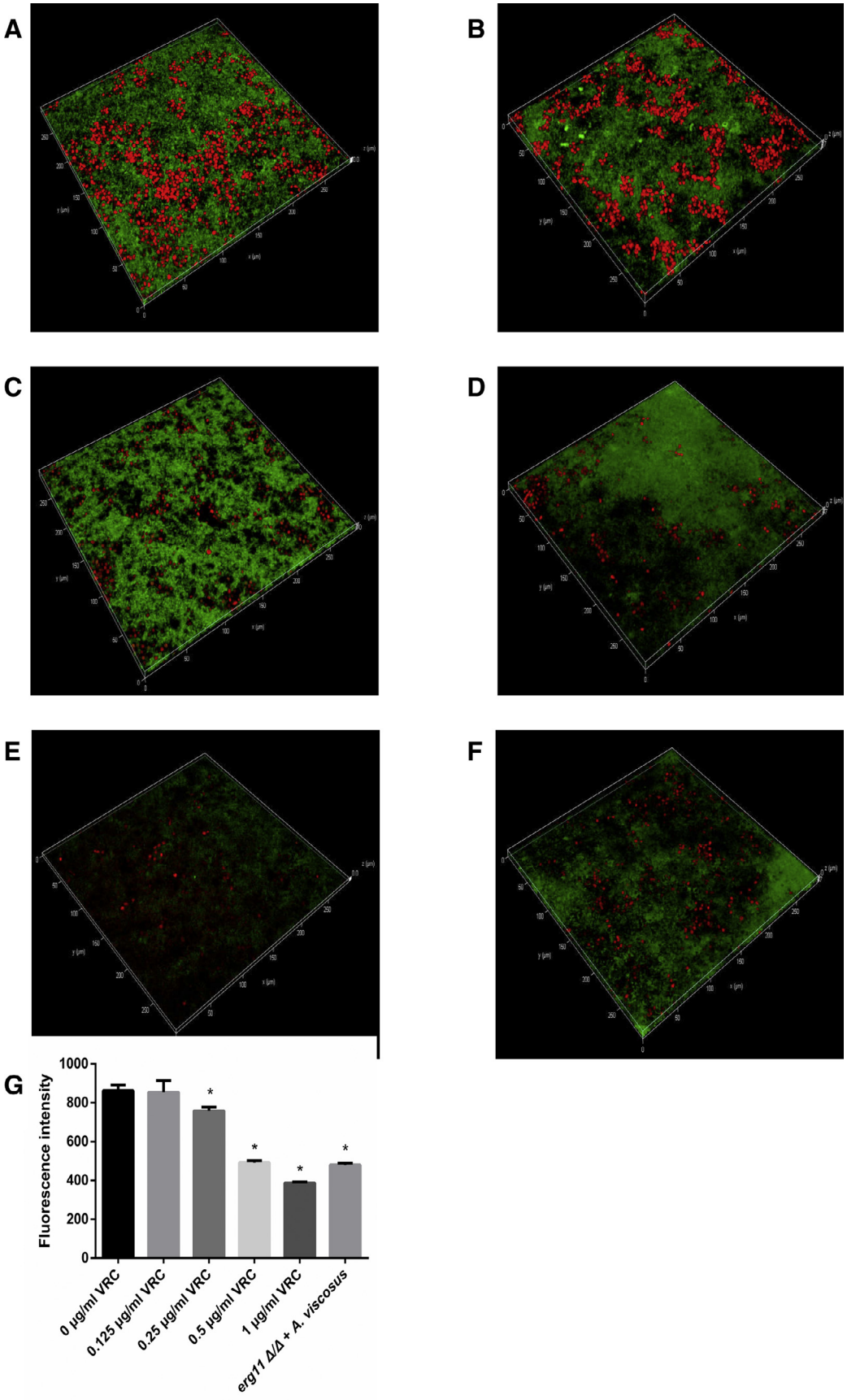

Fig. 5. (A–E) Confocal laser scanning microscopy images of biofilms of *C. albicans* wild-type (labelled red with Cy3) and *A. viscosus* (labelled green with fluorescein isothiocyanate) treated with voriconazole (VRC): (A) 0 μg/mL VRC; (B) 0.125 μg/mL VRC; (C) 0.25 μg/mL VRC; (D) 0.5 μg/mL VRC; and (E) 1 μg/mL VRC. (F) *C. albicans erg11* Δ/Δ + *A. viscosus* control group. Images were obtained at 60 × magnification. (G) Fluorescence intensity of *A. viscosus* in dual-species biofilms was compared. Results were averaged from at least three randomly selected positions of each sample and are presented as the mean ± standard deviation. * $P < 0.05$.

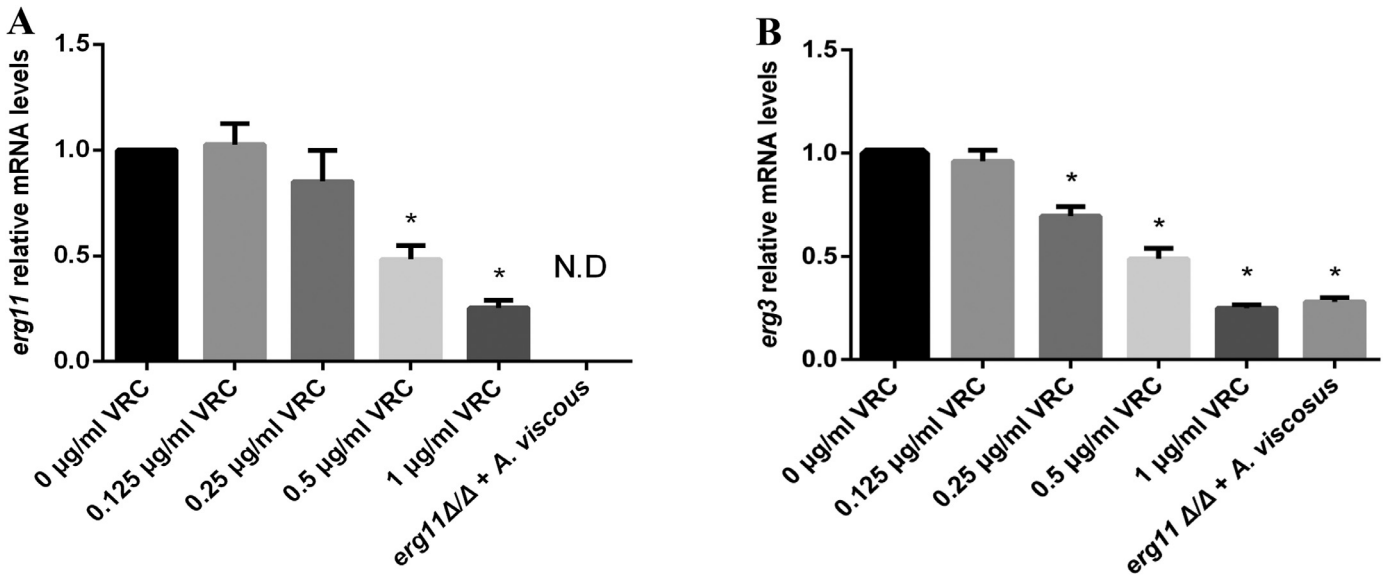

Fig. 6. Quantitative PCR analysis of (A) *ERG11* and (B) *ERG3* mRNA expression in *C. albicans* wild-type *and A. viscosus* dual-species biofilms, showing decreased *ERG11* and *ERG3* expression level in mixed-biofilms. (A) Expression of relative mRNA levels of *ERG11* in all groups. The results showed that the expression of *ERG11* levels decreased remarkably in the 0.5 μg/mL and 1 μg/mL voriconazole (VRC) groups. * $P < 0.05$. (B) Expression of relative mRNA levels of *ERG3* in all groups. The expression of *ERG3* levels decreased remarkably in the 0.25, 0.5 and 1 μg/mL VRC-treated groups. * $P < 0.05$.

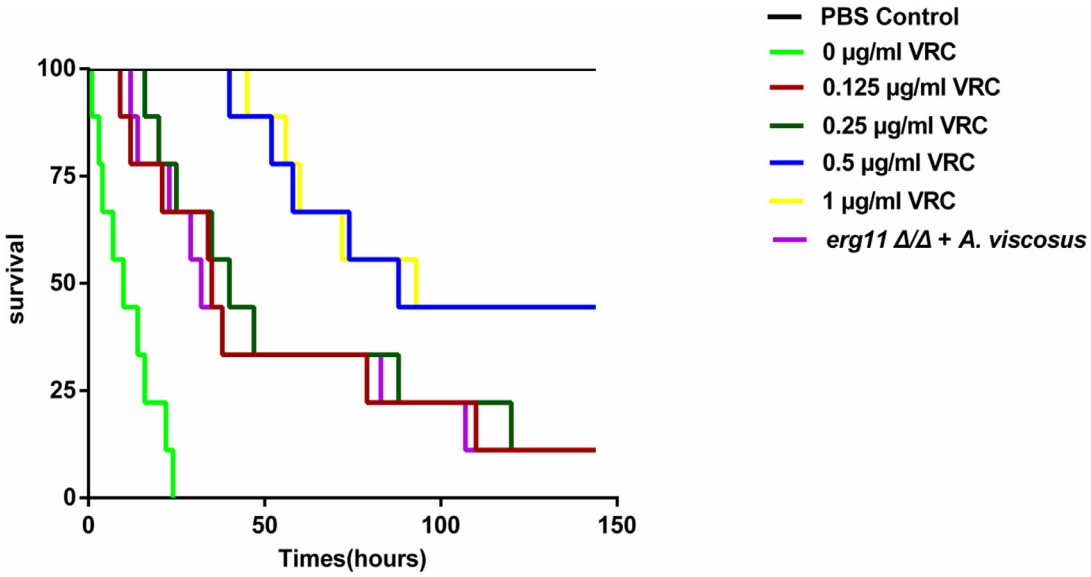

Fig. 7. Survival curves of *Galleria mellonella* larvae treated with voriconazole (VRC). Significant differences were observed between the 0.5 μg/mL and 1 μg/mL VRC-treated groups and the control group (PBS). $P < 0.05$. log-rank test. PBS, phosphate-buffered saline.

4. Discussion

C. albicans and *A. viscosus* are prominent microbes associated with dental root caries [32]. The mechanism of cross-kingdom interactions can provide an important strategy to combat infectious diseases [33]. The current results suggested that a synergistic cross-kingdom interaction occurs in the dual-species biofilms of *C. albicans* and *A. viscosus* owing to increased biomass and virulence. The ergosterol biosynthesis pathway of *C. albicans* was crucial for the interactions, and voriconazole was effective in inhibiting the interaction in the dual-species biofilms *in vitro* and *in vivo*.

Several *C. albicans* mutants co-cultured with *A. viscosus* were used to identify the potential mechanisms by which *C. albicans* promoted the growth of *A. viscosus* in dual-species biofilms. When the *C. albicans phr1*Δ/Δ and *phr2*Δ/Δ mutants were co-cultured with *A. viscosus*, the biomass of the dual-species biofilms was also increased. This indicated that the pH-regulated Rim101p response pathway had no significant effect on the interaction [34]. Ergosterol is an important lipid component in the fungal cell membrane that maintains the normal function of membranes [35]. The *ERG* genes can modulate the target enzymes of the sterol synthesis pathway to regulate ergosterol synthesis, which also plays a key role in coping with external environmental stress [36]. We found that the *C. albicans erg11*Δ/Δ mutant lost the ability to promote the growth of *A. viscosus*, suggesting that the integrity of the *C. albicans* cell membrane is critical for the cross-kingdom interaction. In the future, we will investigate the *A. viscosus* pathways that respond to the *C. albicans* ergosterol pathway to decipher the entire interaction mechanism.

To the best of our knowledge, this is the first study to investigate the pathogenicity of *A. viscosus* combined with *C. albicans* in a *G. mellonella* model. Results of the survival analysis indicated that the pathogenicity of *C. albicans* wild-type and *A. viscosus* combination was significantly higher than that of *C. albicans erg11*Δ/Δ mutant and *A. viscosus* combination in the *G. mellonella* model. Changes in the *C. albicans* cell membrane may decrease the virulence of the dual-species consortium *in vivo*. In the future, we will investigate the dynamic change of virulence factors both from *A. viscous* and *C. albicans* in the mixed biofilms by dual-species transcriptome analysis.

The critical role of the *ERG11* gene of *C. albicans* led us to choose voriconazole, which targets the enzyme encoded by the *ERG11* gene, to treat the dual-species biofilms. Voriconazole is a novel triazole antifungal drug based on the structure of fluconazole. Compared with other triazole antifungal drugs, voriconazole has a broader antifungal spectrum and better efficacy against *Candida* spp. Besides the target in fungi, voriconazole also has an indirect immunomodulatory effect. This indirect mechanism has not been completely elucidated [37]. The current results demonstrated that voriconazole was highly effective in reducing the biomass and virulence of *A. viscosus* when co-cultured with *C. albicans* both *in vitro* and *in vivo*. Voriconazole not only kills *C. albicans* by reducing the amount of ergosterol in the fungal membrane at high concentrations but also causes the accumulation of aberrantly formed sterols with C14-methyl groups, altering the structure of membranes to inhibit *C. albicans* growth at low concentrations [38]. The decrease in the abundance of *Actinomycetes* can cause the transition of root caries from being active to inactive [3]. Therefore, use of an appropriate dose of voriconazole should be an effective method in inhibiting root caries. Interestingly, we also found that voriconazole reduced hyphal formation in polymicrobial biofilms. Morphogenesis is a crucial process during biofilm formation [39], indicating that regulation of the *ERG* genes exhibits cross-interactions with many other pathways, such as those controlling biofilm formation and morphological transformation [38].

The *ERG3* gene is proven to be non-essential, whilst *ERG11* has been shown to be essential in the ergosterol biosynthesis pathway [38,40]. The current findings indicated that voriconazole inhibited the expression of both *ERG11* and *ERG3*, which is in agreement with Nailis et al [41] who also observed that the levels of *ERG11, ERG3* and *ERG1* were downregulated in azole-treated biofilms. The current results demonstrated that voriconazole can downregulate the ergosterol biosynthesis pathway to influence the dual-species biofilms. The different actions of *C. albicans erg11*Δ/Δ and *erg3*Δ/Δ mutants on the growth of *A. viscosus* indicated that the *ERG11* gene may be more important than the *ERG3* gene in the cross-kingdom interactions. We will also focus on the different functions of *ERG11* and *ERG3* genes in the fungi–bacteria cross-kingdom interactions in the future. The results of the current research provide new information on the microbiological and clinical features of root caries and may have relevance for other fungal–bacterial interactions associated with polymicrobial infections in humans as well as new anti-infective drug discovery [4,7,42].

5. Conclusion

Cross-kingdom interactions between *C. albicans* and *A. viscosus* are associated with the development of root caries. The ergosterol pathway of *C. albicans* is critical for the dual-species interaction of *C. albicans* and *A. viscosus*. Voriconazole blocks the cross-kingdom interaction by regulating the ergosterol pathway, indicating its potential capability to treat dental root caries.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.02.010.

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