Int J Clin Exp Pathol 2016;9(11):12057-12067
www.ijcep.com /ISSN:1936-2625/IJCEP0040648

Original Article

Candida albican-bacterial polymicrobial biofilms in recurrent vulvovaginal candidiasis contributes to the drug resistance of the disease: an assessment based on *in vivo* and *in vitro* assays

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Received September 25, 2016; Accepted September 28, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: Ten percentage of vulvovaginal candidiasis (VVC) patients experience development of VVC into recurrent VVC (RVVC), which is caused by incomplete eradication of *Candida* species that form biofilms alone or with bacteria. The purpose of our study was to investigate the effect of types of biofilms on the virulence and antifungal agent resistance of *C. albican* species. The biofilm formation rate and antifungal agent susceptibility of *C. albican* strains isolated from VVC or RVVC patients were assessed. *In vitro* polymicrobial biofilms were induced to assess the association between *C. albican* strains and bacteria species in RVVC. The data of *in vitro* experiments was validated with rat models. Among the 186 *C. albican* strains collected, those isolated from RVVC patients showed higher biofilm formation rates and stronger antifungal agent resistance. Once concatenatedly cultured with bacteria, the polymicrobial biofilms exhibited stronger growth rates than either mono *C. albican* biofilm. And the higher growth rates of polymicrobial biofilms were associated with stronger resistance to all antifungal agents. With rat models, it was found that colony numbers in vaginal samples isolated from rats injected with mixed microbial suspension were significantly higher than those with *C. albican* suspension. The histological examination of vaginal samples showed that rats with polymicrobial biofilm were subjected to heavier RVVC symptoms. Findings outlined in our study revealed the high biofilm formation rate and strong antifungal agent resistance of *C. albican* strains isolated from RVVC and formation of polymicrobial biofilm would strengthen the impairments of *C. albican* on vagina.

Keywords: Antifungal drugs, *Candida albican*, *EGR11*, polymicrobial biofilm, recurrent vulvovaginal candidiasis

Introduction

Vulvovaginal candidiasis (VVC) affects over 75% of females at least once in their entire life, predominantly in their reproductive years [1-4]. Generally, vulvovaginitis are characterized by infections at the vaginal lumen as well as the vulva, symptoms of which include burning, itching, soreness, an abnormal discharge, and dyspareunia [5]. Among those who are impaired by VVC, *ca.* 5-10% patients will experience an episode of acute VVC developing into recurrent VVC (RVVC) [1, 2]. Currently, two main hypotheses have been proposed for the cause of RVVC: (i) reinfection via sexual transmission or from reservoirs in other organs [6]; (ii) relapse due to incomplete eradication of *Candida* species that have increased virulence or are drug-resistant [7, 8]. And with the development of theories and knowledge related to RVVC, the second causal hypothesis has drawn emerging attention of scientists and clinicians these years [9, 10].

Although several *Candida* species are reported to be involved in the attack of VVC and RVVC [9], *Candida albicans* is proved to be the predominant aetiological cause of vaginitis with a prevalence of 70-90% in various reports and expressing mechanisms which allow the coloni-

zation or the infection of the host [4]. By employing techniques of scanning electron and confocal microscopy, it is found that wild-type *C. albicans* strains are capable of forming biofilms on the vaginal mucosa *in vivo* and *in vitro* as indicated by high fungal burden and microscopic analyses [10]. These biofilms are defined as highly organized sessile microbial communities of bacteria, fungi, or both that attach to abiotic or biotic surfaces, forming a complex three-dimensional architecture [11]. The structure is of remarking medical significance in that biofilms suppress susceptibility of pathogens to antibiotic agents and enhance the spread of antibiotic resistance [12]. Furthermore, biofilms also provide a shelter for opportunistic pathogens to thrive and become a resource of infection [13]. Except for the complicated physical structure, biofilms are generally polymicrobial in nature, consisting of members of the endogenous microbiota as well as nosocomial pathogens, which make biofilms even more difficult to be diagnosed as well as being treated [5]. For *C. albicans* in RVVC, its interaction with other microbes has also been verified. As reported by Esim Buyukbayrak *et al.* [14] and Buchta *et al.* [15], approximately 20-34% of RVVC samples are impaired by vaginal bacterial pathogens such as *Streptococcus agalactiae* and *Gardnerella vaginalis*. And another survey of biofilms formed on intrauterine devices (IUDs) confirmed the presence of multiple bacterial pathogens along with *C. albicans* [16]. Compared with monomicrobial biofilms, polymicrobial biofilms represent a stronger potential to serve as infectious reservoirs for a variety of microorganisms and cast more severe issues to public health.

In recent years, with the universal application of azole drugs, the resistance of *C. albicans* against anti-fungal agents dramatically increases, which results increased incidence of infection of *C. albicans* and mixed microbes infected RVVC patients, leading to harder treatment of the disease. And given the crucial function of polymicrobial biofilms in the drug resistance of microbes, it is reasonable to conduct a comprehensive investigation on the microbe composition in RVVC samples and to study the biofilm formation potential of *C. albicans* along with other microbes. Thus, in the current study, the antifungal agents resistance of *C. albicans* isolated from 186 RVVC patients in Wenzhou, China were assessed. The biofilm formation potential of *C. albican* strain C54 as well as C54 strain with bacteria species and the anti-fungal agents resistance of different types of biofilms were detected with a series of *in vitro* and *in vivo* assays. The current study is not only a supplement to the microbe composition of RVVC from different districts but also provides a reference for the application of antibiotic agents for treatment of VVC or RVVC.

Materials and methods

Patients and sample collection

One hundred and eighty six female VCC patients were enrolled in the current study for isolation of *C. albicans* from 2013 June to 2015 June in The First Affiliated Hospital of Wenzhou Medical University. Patient involved should meet the following criteria: 1) Cases involved in the assays should be available to detailed information of clinicopathological and prognostic characteristics. 2) Patients with vaginal secretion being confirmed to contain blastospore or pseudohypha were diagnosed as VVC. 3) Patients attacked by VVC for more than four times one year were diagnosed as RVVC. Patients those were previously treated with anti-fungal drugs within one year were excluded from the study. The vaginal secretion of all the patients were collected from posterior vaginal fornix or vaginal wall by senior clinicians and preserved for subsequent isolation of *C. albicans*. After isolation of *C. albicans*, patients were treated with routine VVC therapies and were followed up for follow-up six months to determine the recurrence of VVC.

The study was approved by The First Affiliated Hospital of Wenzhou Medical University ethnics committee. The ethics committee approved the relating screening, inspection, and data collection of the patients, and all subjects signed a written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

C. albicans control strain and animals

C. albicans strain ATCC14053 were provided by American Type Culture Collection (ATCC) and cultured routinely. Six week old female SPF ICR mice (weighting 20.0 ± 2.0 g) were purchased from Wenzhou Medical University and maintained in cages at room temperature (20-25°C)

Table 1. Demographic information of VVC patients in the current study

	Patient type		*P* value
	VVC	RVVC	
Patients number	84	102	-
Age	34.2	33.1	0.26
Strain number	84	102	-
Recurrent rate (%)	17.9	38.2	0.01*

"*", statistically significant difference, $P < 0.05$.

with a constant humidity (55 ± 5%) with free access to food and water in a 12:12-h light-dark cycle. All animal experiments were conducted in the accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of Laboratory Animals of The First Affiliated Hospital of Wenzhou Medical University.

Identification of C. albicans

Fungal isolates from different vaginal secretion samples were cultured on Sabouraud medium at 28-35°C for 4 h before selecting colonies for Gram staining. And yeast-like fungi were transferred to CHROMagar Candida and cultured at 35°C for 24 h. Identification of *C. albicans* were performed based on the colony morphology, colony color, Gram staining results, lamydospore, germ tube formation assay of isolates as well as the identifying results of VITEK-2 identification system (bioMérieux).

Induction of biofilms in vitro

Biofilms of different *C. albicans* strains were induced by incubating different strains onto 1 cm×1 cm coverslips placed in six-well plates ($0.5\text{-}2.5\times10^6$/each well) and cultured at 37°C for 90 min. Thereafter, cells not adhere to the coverslip were removed. Then 3 mL YDB medium was added into each well and cells were cultured at 37°C for 72 h.

Monomicrobial biofilms or polymicrobial combinations for subsequent assays were classified as following: A) C54 group, monomicrobial biofilms formed by *C. albicans* C54 strain. B) C54 + *Escherichia Coli group*, polymicrobial biofilms formed by *C. albicans* C54 strain and *E. Coli* together. C) C54 + *Streptococcus agalactiae* group, polymicrobial biofilms formed by *C. albicans* C54 strain and *S. agalactiae* together. And induction of biofilms was conducted as described above.

Induction of vulvovaginal candidiasis in vivo

To induce VVC in ICR mice, different combinations of microbes were injected into the vagina of mice: A) Control group, mice were injected with 50 μL normal saline for 5 days, each time per day. B) ATCC14053 group, mice were injected with 50 μL *C. albican* ATCC14053 suspension (10^8 cfu/mL) for 5 days, each time per day. C) C54 group, mice were injected with 50 μL *C. albicans* C54 suspension (10^8 cfu/mL) for 5 days, each time per day D) C54 + *E. Coli group*, mice were injected with 25 μL *C. albicans* suspension (10^8 cfu/mL) and 25 μL *E. Coli* suspension (10^8 cfu/mL) for 5 days, each time per day. E) C54 + *S. agalactiae* group, mice were injected with 25 μL *C. albicans* suspension (10^8 cfu/mL) and 25 μL *S. agalactiae* suspension (10^8 cfu/mL) for 5 days, each time per day. Upon completion of administration, vaginal secretion of mice was collected. Then the mice in different groups were treated with 50 μL normal saline, 50 μL 1 mg/mL itraconazole, 50 μL 1 mg/mL itraconazole and 4 mL/mL *Lactobacillus* every day for 10 days, respectively. Thereafter, vaginal secretion of mice in different groups were collected for determining the colony numbers and mice were sacrificed to collect vaginal tissue for subsequent histological detection. Each group was represented by at least five replicates.

Crystal violet assay

Biofilm biomass of different groups cultured 72 h was quantified using the crystal violet assay, as previously described [17]: briefly, every 24 h, dried cover slips of different groups were placed in 110 μL 0.4% crystal violet at 37°C for 45 min at 175 rpm. Then 200 μL 95% alcohol was added and coverslips were placed at 37°C for 45 min for decolorization. The formation of biofilms was detected by detecting OD_{595} value using a Microplate Reader.

Assessment of antifungal drug susceptibility

Antifungal drug susceptibilities of *C. albicans* isolates and different biofilms were assessed using ATB-fungus 3 kit (BioMerieux, France) according to the manufacturers' protocol.

Scanning electron microscopy (SEM) and confocal microscopy (CM) detection

The ultrastructure of different biofilms was detected using SEM and CM according stan-

Table 2. The antifungal drug susceptibilities of all the strains

Susceptibility	Antifungal drug				
	Amphotericin B	Itraconazole	Voriconazole	5-fluorocytosine	Fluconazole
S	185 (99.46%)	172 (92.47%)	179 (96.24%)	183 (98.39%)	180 (96.77%)
R	1 (0.54%)	8 (4.30%)	4 (2.15%)	2 (1.08%)	4 (2.15%)
M	0 (0.00%)	6 (3.23%)	5 (2.69%)	1 (0.54%)	2 (1.08%)

S, sensitive to the drug. R, resistant to the drug. M, medium to the drug.

dard procedure every 24 h, respectively. For SEM detection, observation was conducted using in a Hitachi S-2700 scanning electron microscope (LSUHSC Imaging Core) at 1000× magnification. For CM detection, samples were examined under oil immersion with a Nikon Eclipse E800 microscope with Metamorph software at 600× magnification.

PCR validation

For PCR detection, total DNA in six *C. albicans* isolates [including standard *C. albicans* strain ATCC14053 (sensitive), *C. albicans* strain C32 (sensitive), VVC *C. albicans* strain C16 and C47 (resistant), RVVC *C. albicans* strain C27 and C65 (resistant)] was extracted using Total DNA Extraction Kit according to the manufacturer's instruction (Calt. No. DP305-02, TIANGEN, Beijing, China). The final PCR reaction mixture of volume 25 μL consisted of 2.5 μL of 10× PCR buffer, 2 μL dNTP (2.5 mM), 0.25 μL rTaq, 2 μL of each primers (*ERG11*, forward: 5'-GGGAAAGTTTCTAAAGGGG-3', reverse: 5'-TATGTTAATCCAACTAAGTAA-3'), 0.5 μL of the cDNA template, and 16.75 μL of ddH_2O. Amplification parameters were as follows: denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 1 min, and at 72°C for 10 min. Expression status of *ERG11* in different isolates were detected using gel electrophoresis.

H&E staining

The histological changes in sections of vaginal tissues from different groups were observed using H&E staining [Hematoxylin and eosin (H&E) staining protocol]: briefly, tissues were placed into Bouin solution (4% formaldehyde) for perfusion fixation. Following this, they were dehydrated using different concentration of alcohol and vitrified in dimethylbenzene. Samples were embedded in paraffin, sectioned and stained with H&E and the results were detected under a microscope at 200× magnification.

Statistical analysis

All the data were expressed in the form of mean ± SD. one-way ANOVA and post doc test by Dunnett method were performed using general liner model. The difference between recurrent rates of VVC was examined using Chi-square test. Statistical significant was accepted with P value < 0.05. All the statistical analyses were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA).

Results

Antifungal drug susceptibility of C. albicans strains

In the current study, 186 female VVC patients were enrolled for isolation of *C. albicans*. In total, 186 isolates of *C. albicans* strains were identified from the vaginal secretion of all these patients, among which 102 strains were isolated from RVVC patients and 84 strains were isolated from VVC patients (**Table 1**). The average age of the subjects was 33.6 years old, ranging from 20 years old to 62 years old. The average age of VVC patients was 34.2 years old and the average age of RVVC patients was 33.1 years old, no significant difference was detected between VVC and RVVC patients for age (P = 0.26). For recurrent rate, the value of VVC patients was 17.9% while the value of RVVC patients was 38.2%, the difference between recurrent rates was statistically significant (P = 0.01), which was indicative of the stronger pathogenicity of RVVC *C. albicans* strains.

The antifungal drug susceptibilities of all the strains were assessed using ATB-fungus 3 kit and the detail results were shown in **Table 2**. It was found that itraconazole was the azole drug which was easiest for *C. albicans* strains to develop resistance. And based on the results of antifungal drug susceptibility, five in all the strains showed drug resistance to at least two types of antifungal drugs (**Table 3**). For subse-

Table 3. Detail information of five *C. albicans* that were resistant to at least two antifungal drugs

Strain ID	Patient age	VVC type	Susceptibility to antifungal drugs					Growth rate of biofilm (OD_{595})		
			Amphotericin B	Itraconazole	Voriconazole	5-fluorocytosine	Fluconazole	24 h	48 h	72 h
C12	48	Non-recurrent	S	R	R	S	R	0.29	0.33	0.35
C27	23	Recurrent	S	R	R	S	R	0.26	0.49	0.61
C45	51	Recurrent	S	R	R	S	R	0.25	0.38	0.46
C46	23	Recurrent	S	R	R	S	R	0.39	0.41	0.52
C54	44	Recurrent	R	R	S	S	S	0.32	0.41	0.64

S, sensitive to the drug. R, resistant to the drug.

Table 4. MIC values of different microbe combinations in both free and biofilm forms

Strain	Antifungal drug type							
	Amphotericin B (μg/mL)		Itraconazole (μg/mL)		5-fluorocytosine (μg/mL)		Fluconazole (μg/mL)	
	Free	Biofilm	Free	Biofilm	Free	Biofilm	Free	Biofilm
ATCC14503	0.5	32	0.03	4	2	64	2	32
C32	1	32	0.06	8	4	128	4	512
C54	8	128	4	128	64	512	128	1024
C54 + *E. coli*	16	512	16	≥ 256	128	≥ 512	512	≥ 1024
C54 + *S. agalactiae*	16	≥ 512	8	≥ 256	128	≥ 512	512	≥ 1024

quent assays, strain C54 was selected based on its OD_{595} value at 72 h (**Table 3**).

Furthermore, the MIC values of *C. albicans* strain ATCC14053, susceptible strain C32, resistance strain C54, C54 + *E. coli*, and C54 + *S. agalactiae* were measured with both their free and biofilm forms. As shown in **Table 4**, formation of biofilms dramatically increased the values of MIC of all the treatments and co-culture of fungal and bacteria strains also further enhanced the drug resistance of C54 strain. Moreover, the strongest resistance against each type of antifungal drug was observed for the two types of polymicrobial biofilms, which confirmed the key role of polymicrobial biofilms in promoting the antifungal drug resistance of *C. albicans* in VVC.

Biofilms formation potential of different C. albicans strains

All the *C. albicans* strains were capable of forming biofilms during 72 h culture (**Figure 1A**). Based on the results of violet crystal assays, the average OD_{595} value of resistant strains were higher than that of non-resistant strains at all the three sampling points, and the difference was statistically significant (**Figure 1B**) ($P < 0.05$), representing the stronger potential of resistant *C. albicans* strains to form biofilm *in vitro*. However, analysis based on resources of vaginal secretion indicated that strains isolated from RVVC samples formed biofilm at a similar rate compared with strains isolated from VVC samples (**Figure 1C**). In addition, it was found that among the resistant strains, the biofilm formation potential was also similar: strains resistant to two at least two types of antifungal drugs showed an identical biofilm forming rate compared with those only resistant to one type of antifungal drug during the early stage of biofilm formation (**Figure 1D**). The difference of biofilm formation potential between resistant and non-resistant strains isolated from RVVC samples was analyzed as well and the results indicated that although all isolated from RVVC vaginal secretion, significant distinct in the biofilm formation capability could be detected between resistant and non-resistant strains (**Figure 1E**) ($P < 0.05$). Moreover, even though the biofilm formation rate of C54 strain was higher than any other *C. albicans* strains (**Table 3**), its biofilm growth rate was significantly lower than any polymicrobial biofilm (**Figure 1F**).

Ultrastructure of different biofilms

Ultrastructure of different biofilms was detected using both SEM and CM post-inoculation for

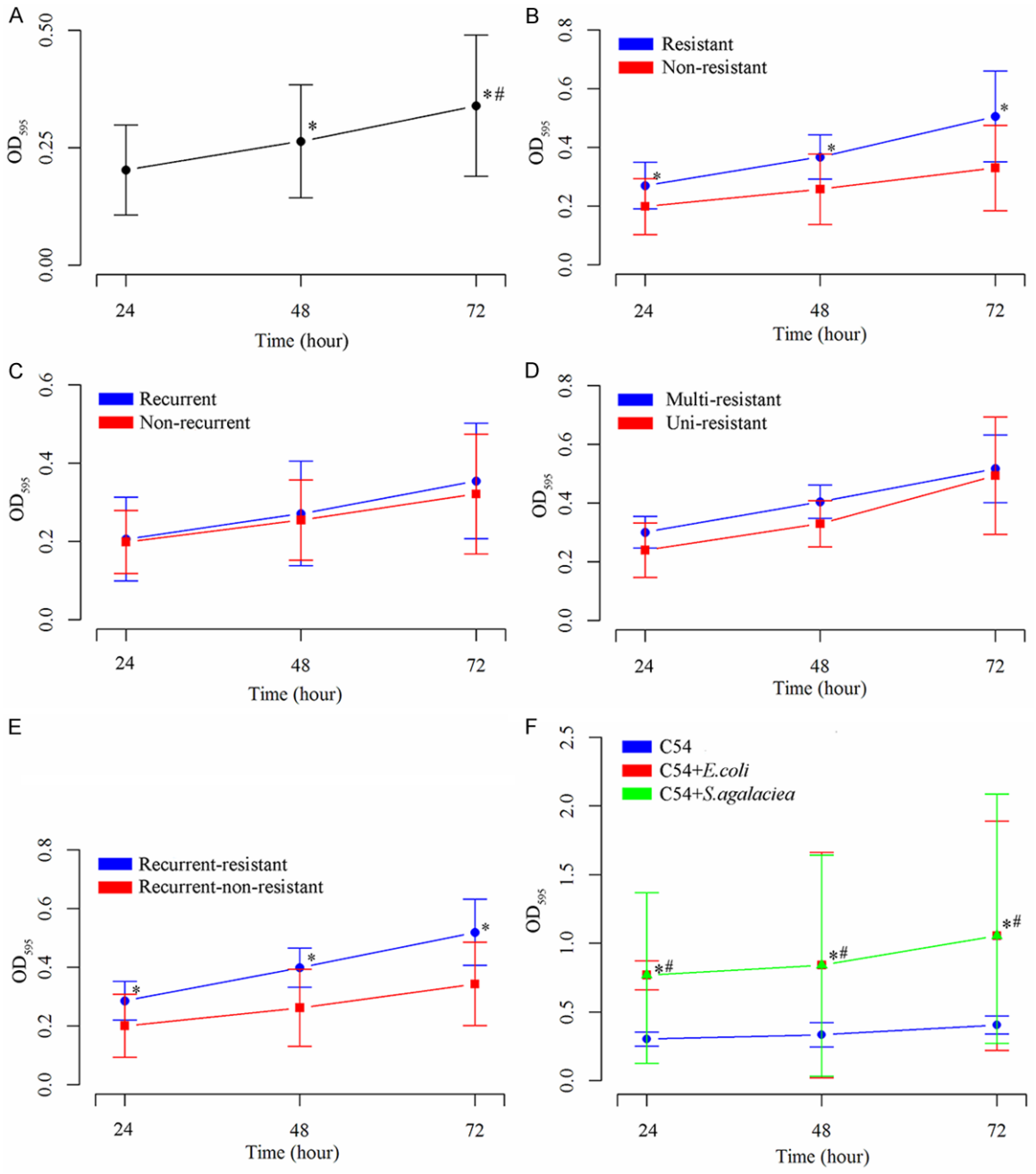

Figure 1. Quantitative analysis results of biofilm formation rates of different types of *C. albican* strains. A: Average OD_{595} values of all the *C. albican* strains in 24, 48, and 72 h culture. "*", significantly different from 24 h, $P < 0.05$. "#", significantly different from 48 h, $P < 0.05$. B: Average OD_{595} values of antifungal drug resistant or antifungal drug non-resistant *C. albican* strains in 24, 48, and 72 h culture. "*", significantly different from non-resistant *C. albican* strains, $P < 0.05$. C: Average OD_{595} values of recurrent or non-recurrent *C. albican* strains. D: Average OD_{595} values of muti-resistant or uni-resistant *C. albican* strains. E: Average OD_{595} values of recurrent-resistant or recurrent-non-resistant *C. albican* strains, significantly different from recurrent-non-resistant *C. albican* strains. F: Average OD_{595} values of C54, C54 + *E. coli*, or C54 + *S. agalaciea*. "*", significantly difference between C54 and C54 + *E. coli*, $P < 0.05$. "#", significantly difference between C54 and C54 + *S. agalaciea*, $P < 0.05$.

biofilm formation. SEM analysis revealed fungal colonization, the presence of a complex network of both yeast and hyphae, and ECM accumulation *in vitro*. And the density of yeast and hyphae increased with time (**Figure 2A**). Compared with monomicrobial biofilms, the

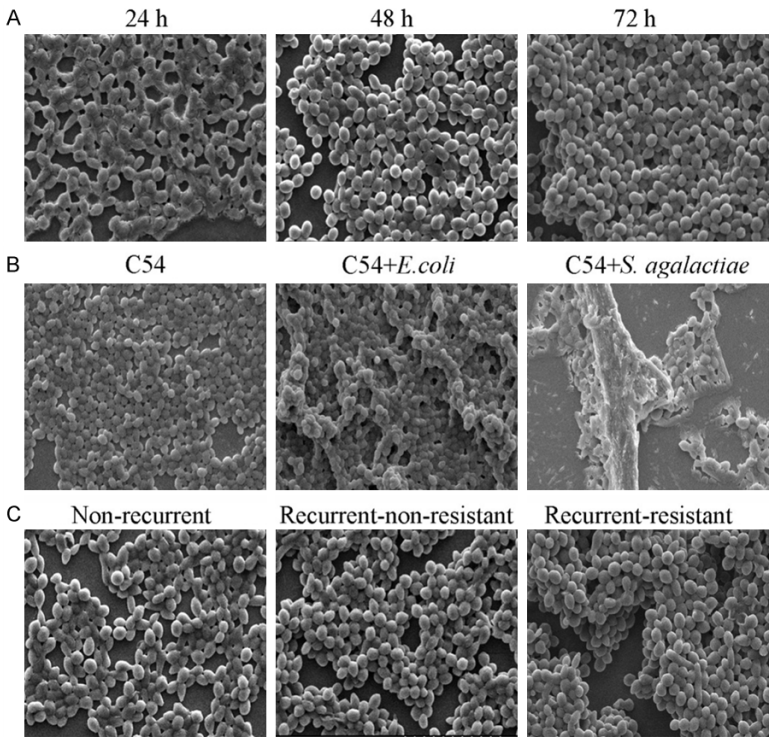

Figure 2. Ultrastructure of different biofilms detected using SEM. A: SEM detected ultrastructure of biofilms formed by *C. albican* strain C54. The density of yeast and hyphae increased with time and a complex network consisted of yeast and hyphae as well as ECM accumulation was observed. B: SEM detected ultrastructure of biofilms formed by C54, C54 + *E. coli*, or C54 + *S. agalaciea*. Compared with monomicrobial biofilms, the structure of polymicrobial biofilms was even more complicated with bacteria covering the biofilm of *C. albicans*. C: SEM detected ultrastructure of biofilms formed by *C. albican* strains with different antifungal drug resistance. Biofilms of resistant *C. albicans* strains were more condensed than those of susceptible strains. Magnification, 1000×.

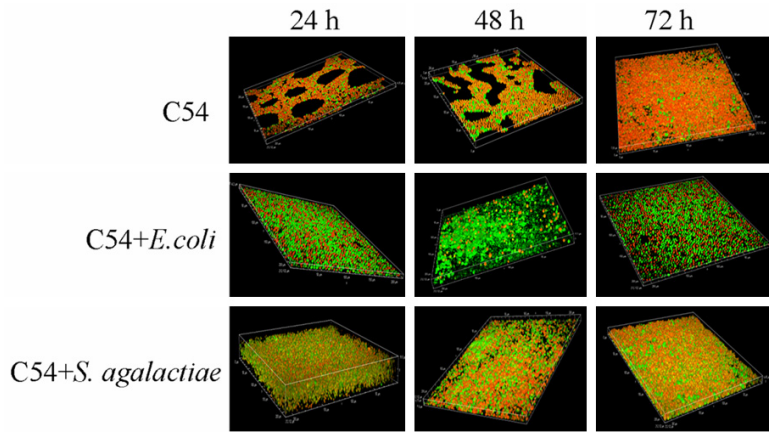

Figure 3. Ultrastructure of different biofilms detected using CM within 72 h. The detection confirmed that the present of ECM as illustrated by the bright red cell-associated staining and the hazy diffuse red material covering the hyphae. Magnification, 600×.

structure of polymicrobial biofilms was even more complicated with bacteria covering the biofilm of *C. albicans* (**Figure 2B**). And biofilms of resistant *C. albicans* strains were more condensed than those of susceptible strains (**Figure 2C**).

CM analysis confirmed that biofilm ECM was present, as demonstrated by the bright red cell-associated staining and the hazy diffuse red material covering the hyphae (**Figure 3**). The thickness of biofilms in different groups were shown in Table S1, and based on the data, the biofilm growth rate detected by CM was similar to that of SEM with biofilms formed by mixed microbes showing higher growth rate.

Expression status of EGR11 gene was enhanced in resistant C. albicans strains

The expression status of *EGR11* gene was detected with six *C. albicans* strains. As illustrated in **Figure 4**, it was found that expression of *EGR11* gene could barely be detected with strains ATCC-14053 and C32 which were antifungal drug sensitive. For antifungal drug resistant strains, the expression of *EGR11* gene was observed for all the four strains. Moreover, the bands for RVVC strains were clearer than those of VVC strains. However, no quantitative analyses were conducted in the current study, which made our conclusion rendered less solid.

Biofilms formed by RVVC C. albicans strain and mixed microbes showed higher growth rate in vivo

The growth rates of biofilms formed by vaginal secretion collected from experimental mice were assessed by violet crystal assay. And identical with the results of *in vitro* assay,

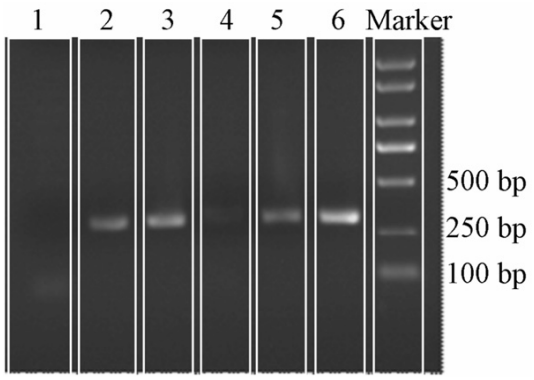

Figure 4. Expression status of EGR11 gene in different C. albican strains. Panel 1, C. albican strain C32 (azole sensitive). Panel 2, C. albican strain C16 (azole resistant). Panel 3, C. albican strain C27 (RVVC azole resistant). Panel 4, standard C. albican strain ATCC14053 (azole sensitive). Panel 5, C. albican strain C47 (azole resistant). Panel 6, C. albican strain 65 (RVVC azole resistant).

biofilms formed by mixed microbial cultures showed significantly higher growth rate than standard *C. albicans* strain (**Figure 5**) ($P < 0.05$). The strongest biofilm formation potential was also observed with mixed microbial cultures (**Figure 5**). However, different from the results of *in vivo* assay, the biofilm formation rate between C54 and ATCC14053 was within similar magnitude. After 10-day treatment of different methods, the colony formation numbers of different vaginal secretion samples were determined. And for mice treated with 50 μL 1 mg/mL itraconazole, no colony could be observed for Control and ATCC14053 groups (**Table 5**) while a large amount of colony were recorded for the other three groups (**Table 5**). For mice treated with 50 μL 1 mg/mL itraconazole and 4 mg/mL *Lactobacillus*, the interesting result was than mice in C54 group showed stronger resistance to the treatment compared with those in *C. albicans* + *E. coli* group, which might infer the sensitivity of polymicrobial biofilms to some specific treatment modalities. Additionally, the histological changes of the mice under different treatments were detected with H&E. And the results of colony formation number were further confirmed by H&E staining: injection of ATCC14053 and C54 group suspension induced proliferation of interstitial fibrous tissue, lymphocytes infiltration, and epithelial cornification in vaginal tissues (**Figure 6**), but the condition of C54 group was more severe than ATCC14053 group. And for groups injected with mixed microbes, the symptoms was even worse when compared with C54 group: purulent inflammation, proliferation of interstitial fibrous tissue, fall-off of and erosion of epithelium could be clearly observed for vaginal tissues collected from C54 + *E. Coli* and C54 + *S. agalactiae* groups. Additionally, concatenated treatment with itraconazole and *Lactobacillus* achieved better outcomes than treatment solely with itraconazole in all the VVC induced groups.

Discussion

The description of biofilms can be traced back to the "little living animalcules, very prettily a-moving" in the plague of Antonie van Leeuwenhoe's teeth in 1683, but the concept of biofilm growth was not officially proposed until 1978 [11]. And with the help of multiple sophisticated microscopic techniques, biofilms are now characterized as well organized microbial communities adhering to an inanimate or living tissue surface [18]. It is generally recognized that attachment to an interface of these microbes is the initiating event in the biofilm process, which will trigger the subsequent self-production of ECM as well as an alteration with respect to the growth and gene regulation of the microbes, leading to reduction of susceptibility to antimicrobial agents and spread of antimicrobial resistance [19]. Therefore, formation of biofilm is of great medical significant in multiple infectious diseases, especially in their persistence and recurrence [20]. As such, biofilms formed by polymicrobes including endogenous microbiota as well as nosocomial pathogens are of greater interest to scientists and clinicians in that polymicrobial biofilms possess stronger potential to resist anti-microbe drugs, i.e., biofilms formed by *Candida spp* and relevant bacteria species in RVVC represents the virulence attributes and mechanism of resistance [5]. Based on the above information, the major purpose of the current study was to investigate the impact of polybiofilms on the management and treatment of RVVC and highlight the emergency need for development of novel therapeutic strategies targeting vaginal biofilms.

C. albicans strains were isolated from 186 VVC patients. Thereafter, the susceptibility to antifungal drugs of all the strains was tested. It was found that strains isolated from RVVC patients

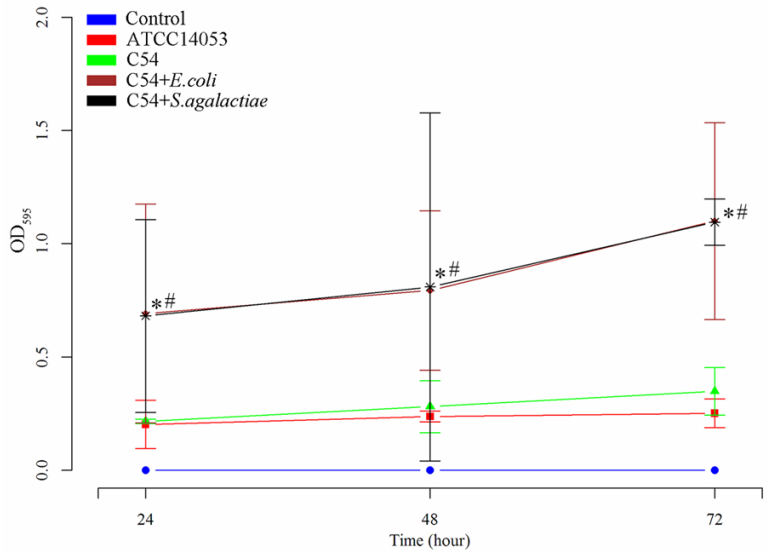

Figure 5. Quantitative analysis results of biofilms formation rates by vaginal secretion collected from different experimental mice. Biofilms formed by mice secretion injected with C54 + *E. coli* or C54 + *S. agalaciea* suspension showed significantly higher growth rate than standard *C. albicans* strain. "*", significant difference between C54 + *E. coli* and ATCC14053 groups, $P < 0.05$. "#", significant difference between C54 + *S. agalaciea* and ATCC14053 groups, $P < 0.05$.

Table 5. Colony formation capability (cfu/mL) of vaginal secretion samples collected from mice treated with different microbe combinations

Group	Treating method		
	Saline	Itraconazole	Itraconazole + *Lactobacillus*
Control	8.25×10^5	0	0
ATCC14053	3.01×10^7	0	0
C54	1.88×10^8	4.30×10^3	1.69×10^2
C54 + *E. Coli*	5.14×10^9	9.12×10^3	3.26×10^2
C54 + *S. agalactiae*	7.09×10^9	7.56×10^3	0.23×10^2

possessed a higher chance to resist more antifungal drugs, which was associated with the up-regulated expression of *EGR11* gene. The *EGR11* gene encodes lanosterol demethylase which is the target of the azole antifungals [21]. The augmented expression of *EGR11* in RVVC was representative of the existence of some mechanism which blocked the inhibition of azole on *EGR11* gene and resulted in resistance. However, restricted by experimental designs and instruments, the current study failed to conduct more comprehensive work focusing on the signaling transduction changes that modulated azole resistance between VVC and RVVC *Candida* strains. Additionally, the *in vitro* biofilm formation rates of *C. albicans* strains were also measured, strains isolated from RVVC patients or resistant to multiple antifungal drugs were capable of forming biofilms more quickly. Based on the results of antifungal drug susceptibility assay and biofilm formation rate of different *C. albicans* strains, strain C54 which were resistant to multiple antifungal drugs and possessed the highest biofilm formation rate was selected for subsequent experiments.

Induction of monomicrobial and polymicrobial biofilms *in vitro* clearly illustrated the distinct biofilm formation rates: mixed microbe combinations showed higher biofilm growth rates. Furthermore, the higher biofilm formation rates of polymicrobes were associated with higher resistance to antifungal drugs. The MIC values of both types of polymicrobial biofilms to four antifungal drugs were much higher than the *C. albicans* strain C54. Such results implicated the synergistic interaction between the members involved in the polymicrobial biofilms. Moreover, different microbe treatments were employed on rats to induce biofilm formation *in vivo*. Identical with *in vitro* group, rats injected with mixed microbe suspension were subjected to more severe vaginal impairments and showed stronger resistance to different treating therapies. In reality, in the biological environment of host, *C. albicans* are often to form polymicrobial biofilms with different bacterial species. And within those biofilms, extensive interspecies interactions are likely to take place and influence the transition of *C. albicans* between virulent and nonvirulent states [22]. More surprisingly, studies regarding drug susceptibility indicated that the fungi species existing in polymicrobial biofilms may modulate the action of antibiotics on bacteria and bacteria species can conversely impact the antifungal activity [23]. It is commonly recognized that in a polymicrobial biofilm, fungi and bacteria species employ a called 'quorum-sensing' (QS) systems, which will promotes collective behavior of each species and enhances

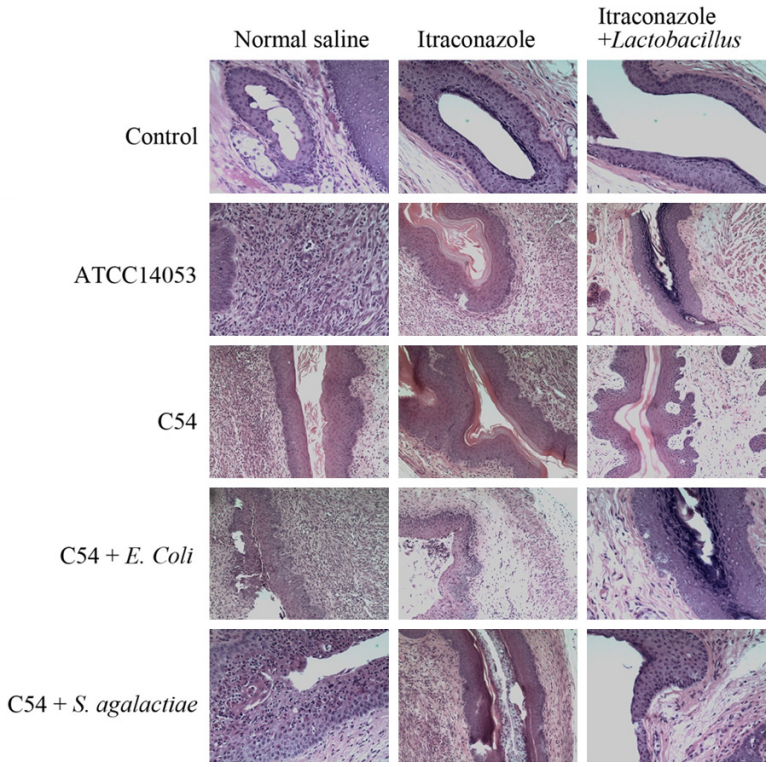

Figure 6. H&E staining of histological changes of the vaginal samples under different treatments. Injection of ATCC14053 and C54 group suspension induced proliferation of interstitial fibrous tissue, lymphocytes infiltration, and epithelial cornification in vaginal tissues. Groups injected with mixed microbe showed purulent inflammation, proliferation of interstitial fibrous tissue, fall-off of and erosion of epithelium Concatenated treatment with itraconazole and *Lactobacillus* achieved better outcomes than treatment with itraconazole. Magnification, 200×.

opportunities to nutrients and shelters [24-26].

In conclusion, findings outlined in the current study revealed the high biofilm formation rate and strong antifungal drug resistance of *C. albican* strains isolated from RVVC patients. Polymicrobial biofilm formed by *C. albican* and bacteria species dramatically increased the biofilm potential of *C. albican* strains and antifungal drug resistance both *in vitro* and *in vivo*, indicating that formation of polymicrobial biofilm can be a severe issue for public health. Therefore, prospective studies are promptly demanded to determine the optimal therapy for RVVC patients impaired by polymicrobial biofilm.

Acknowledgements

This work was supported by grants from the Medical and Health Project of Zhejiang Province (grant 2016KYA137), Zhejiang Provincial Natural Science Foundation of China (grant LY15H190008), Wenzhou Public Welfare Science and Technology Project (grants Y20140707, Y2014-0110), and the Incubation Project of the First Affiliated Hospital of Wenzhou Medical University (grant FHY2014-009).

Disclosure of conflict of interest

None.

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Table S1. Thickness (μm) of biofilms formed by different microbes as detected by CM

Biofilm type	Time		
	24 h	48 h	72 h
C54	4.4	5.4	7.1
C54 + *E. coli*	4.2	11.1	19.5
C54 + *S. agalactiae*	4.2	6.2	6.5