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Influence of culture conditions for clinically isolated non-*albicans Candida* biofilm formation

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ABSTRACT

Non-*albicans Candida* species have been isolated in increasing numbers in patients. Moreover, they are adept at forming biofilms. This study analyzed biofilm formation of clinically isolated non-*albicans Candida*, including *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis* under the influence of different growth media (RPMI 1640, YPD and BHI) and several culture variables (inoculum concentration, incubation period and feeding conditions). The results showed that culture conditions strongly influenced non-*albicans Candida* species biofilm formation. YPD and BHI resulted in larger amount of biofilm formation with higher metabolic activity of biofilms. Furthermore, the growth media seems to have varying effects on adhesion and biofilm development. Growth conditions may also influence biofilm formation, which was enhanced when starting the culture with a larger inoculum, longer incubation period and using a fed-batch system. Therefore, the potential influences of external environmental factors should be considered when studying the non-*albicans Candida* biofilms *in vitro*.

1. Introduction

Candida species are opportunistic pathogens, which are able to cause infections in immunocompromised patients. The *Candida* species are also adept at forming biofilms (Brighenti et al., 2014; Tan et al., 2016). Normally *Candida albicans* is the major pathogens for candidiasis, but recently, more clinical attention has been attracted on infections caused by non-*albicans Candida* species, which have been identified as the infecting pathogens (Donlan and Costerton, 2002; Douglas, 2003). Non-*albicans Candida* species also produce virulence factors once attributed to *C. albicans* (Sardi et al., 2013; Deorukhkar et al., 2014). Furthermore, the biofilms with non-*albicans Candida* species have been detected more on the surfaces of implanted devices with the increasing usage of indwelling medical devices in clinical practice (Donlan and Costerton, 2002; Leonhard and Schneider-Stickler, 2015), and associated with a significant increase of infection risk (Visick et al., 2016).

Therefore, assessing the biofilm formation ability of clinical isolates of non-*albicans Candida* species can help us better understand their virulence potential. There are several factors that can influence biofilm formation, such as medium composition (Kucharikova et al., 2011; Lee et al., 2014; Serrano-Fujarte et al., 2015), inoculum concentration (Cotter et al., 2009; Machado et al., 2015), incubation period (Abdallah et al., 2014; Abdallah et al., 2015; Zago et al., 2015), or feeding conditions (Cerca et al., 2004; Rodrigues et al., 2009). Unfortunately, the information regarding *in vitro* non-*albicans Candida* species biofilm quantification under different culture conditions is very sparse.

In this study, the influence of three commonly used media, namely Yeast Peptone Dextrose (YPD), Roswell Park Memorial Institute medium (RPMI 1640) and Brain Heart Infusion Broth (BHI), and several culture variables (inoculum concentration, incubation period and feeding conditions) on non-*albicans Candida* biofilm were assessed *in vitro*.

2. Material and methods

2.1. Strains and growth media

The Candida species used in this study (*Candida tropicalis*, *Candida krusei* and *Candida parapsilosis*) were isolated from explanted dysfunctional voice prostheses of laryngectomized patients in routine follow up examinations. The primary insertion of voice prosthesis in a surgically created tracheoesophageal fistula is a standard method for voice rehabilitation after total laryngectomy. In any case of a prosthesis failure with speaking difficulties and/or aspiration due to transprosthetic or periprosthetic leakage, the voice prostheisis has to be changed in an ambulant setting.

In this study, dysfunctional voice prostheses due to heavy biofilm formation were explanted from the tracheoesophageal fistulas and processed within 24 h. The prostheses were vortexed in 5 ml PBS for 3 min, the microbial specimen were isolated and analyzed on selective agar plates using standard microbiology methods, stored in −80 °C and thawed before use.

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For each experiment, the microorganisms were grown in YPD (Sigma-Aldrich) at 30 °C for 24 h. Dilutions of YPD, RPMI 1640 (Life Technologies) and BHI (Sigma-Aldrich) were used for culturing the biofilms.

2.2. Biofilm formation

Biofilms were formed in 96-well polystyrene plates according to Ramage et al. (Ramage et al., 2001). Briefly, *C. tropicalis, C. krusei* and *C. parapsilosis* were incubated at 37 °C overnight on agar plates. After incubation, cell density was adjusted to OD_{600} 0.1 or OD_{600} 0.01 in RPMI 1640, YPD or BHI.

Inoculum of each strain (100 μl) was added to the flat-bottom 96-well polystyrene plate in quadruplicate. After a period of adhesion (90 min) at 37 °C, non-attached *Candida* cells were removed by two rounds of washing with PBS and fresh media was added. One set of plates was analyzed immediately after the period of adhesion. A second set of plates were incubated at 37 °C for 24 h or 48 h without shaking.

In order to evaluate the effect of fed-batch growth on 48 h biofilms, the culture medium was replaced by a fresh medium after 24 h of growth.

The plates were washed three times with PBS. Biofilms were stained with 150 μl of 0.1% (w/v) crystal violet solution for 30 min, washed and incubated in 200 μl of 30% (v/v) acetic acid for 15 min to extract the crystal violet retained by the cells. The extract was used to determine the amount of biofilm by measuring its A_{590} with a microtiter plate reader. At least six replicates were conducted for each sample, and each experiment was performed at least in triplicate.

2.3. XTT assay

The metabolic activity of biofilms was calculated using a 2, 3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5 carboxanilide (XTT) reduction assay (Nett et al., 2011). Wells were washed once in PBS, and incubated at 37 °C for 2 h with 150 μl XTT working reagent (XTT 180 mg l^{-1}, AppliChem, Darmstadt, Germany); conversion of the XTT substrate to a soluble colored formazan product correlates with cell viability. The resulting absorbance was read at 490 nm.

3. Results

3.1. Biofilm formation by Candida species in different media

After 24 h, all of the *Candida* species formed biofilms in the three different media (Fig. 1). The effect of growth conditions on the ability of *C. parapsilosis* to biofilm was more pronounced (Fig. 1A). When grown in YPD medium, *C. parapsilosis* manifested significantly greater biofilm formation. While *C. krusei* and *C. tropicalis* preferred BHI and YPD media, in which both *C. krusei* and *C. tropicalis* had significantly enhanced biofilm formations compared with RPMI 1640 medium(Fig. 1B and C).

3.2. The metabolic activity of biofilms in different growth media

XTT reduction quantifies the metabolic activity of biofilms with the three species of *Candida* in the different media (Fig. 2). The results show a correlation between biofilm formation and the metabolic activity of biofilms with *C. tropicalis, C. parapsilosis* and *C. krusei*. The metabolic activity of biofilms with *C. parapsilosis* and *C. krusei* demonstrated significantly lower when grown in RPMI 1640 compared to growth in BHI or YPD medium.

3.3. Adhesion assay in different growth media

As shown in Fig. 3, *C. parapsilosis* adhesion properties were independent of the choice of medium (Fig. 3A), whereas *C. krusei* and *C. tropicalis* manifested significantly better adhesion properties in BHI or YPD medium (Fig. 3B and C). However, there were not significant adhesion differences when tested strains were grown in BHI or in YPD medium.

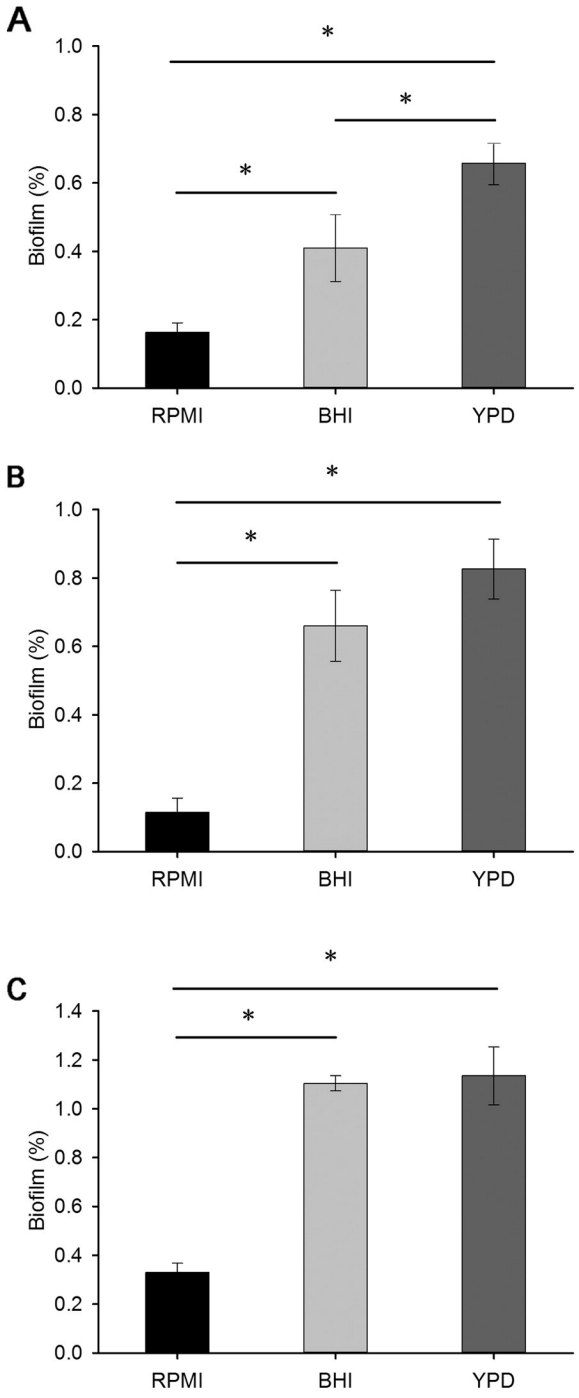

Fig. 1. Biofilm formation by clinically isolated non-*albicans Candida* species in different media. (A) *C. parapsilosis*; (B) *C. krusei*; (C) *C. tropicalis*. The results shown represent the means and standard deviations (error bars) of three independent experiments. Statistical differences in the biofilm formation in different media are marked with * ($p < 0.05$).

Influence of inoculum concentration on biofilm formation.

The inoculum concentration could influence biofilm formation considerably (Cotter et al., 2009). Here, two inoculum concentrations, OD_{600} 0.1 and OD_{600} 0.01, were tested to evaluate the influence of inoculum concentration on biofilm formation. As shown in Fig. 4,