

Evaluation of culture conditions for mixed biofilm formation with clinically isolated non-*albicans* *Candida* species and *Staphylococcus epidermidis* on silicone



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ABSTRACT

Silicone is frequently used in clinical and medical fields for medical devices. Mixed biofilms composed of *Candida* and bacterial species causes frequently failure of medical silicone devices. In this *in vitro* study, we analyzed mixed biofilm formation of clinically isolated non-*albicans* *Candida* species and *Staphylococcus epidermidis*, including *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis* under the influence of different growth media (RPMI 1640, BHI and TSB) and several culture variables (incubation period, feeding conditions and FBS). Our results showed that culture conditions strongly influence mixed biofilm formation. TSB and BHI resulted in larger amount of biofilm formations with stronger metabolic activity of biofilms. Growth conditions may also influence the biofilm formation, which was enhanced by longer incubation period, using a fed-batch system and FBS. Therefore, the potential influences of external environmental factors are very important for mixed biofilm formation with clinically isolated non-*albicans* *Candida* species and *S. epidermidis*, which should be considered when designing or studying the mixed biofilm under *in vitro* conditions.

1. Introduction

A biofilm is described as a well-structured population of microbial cells that are enclosed in a self-produced extracellular polymer matrix and adhere to a surface [1]. These structured communities on medical devices significantly increase the risk of infection [2,3]. Although many implant-associated infections are caused by a single pathogenic microorganism, which have been extensively studied in the past, it has become clear that polymicrobial biofilms are the dominant form in nature. Mixed bacterial-fungal biofilms infections involving medical devices have been attracting more attention [4,5].

Most cases of candidiasis have been attributed to *Candida albicans*, an opportunistic resident species in the oral cavity, but also to non-*albicans* *Candida* species. Furthermore, the non-*albicans* *Candida* species are adept in forming biofilms of medical devices in clinical practice [4,6,7]. *Staphylococci*, especially the species of *Staphylococcus epidermidis*, are known as a most prevalent opportunistic pathogen, which cause the majority of implant-associated infections [8,9]. Notably, approximately 20–40% of cases of candidaemia were accompanied by bacteraemia, with *Staphylococcus* species being the main pathogens [10,11].

Silicone is widely used in clinical and medical fields due to its biocompatibility and mechanical and moulding properties [12,13]. However, silicone becomes rapidly colonized by microorganisms that form a biofilm, which limits the devices life time and increases the risk of infection [5,14]. Although biofilm-associated implant infections involving *Candida* or bacteria are common, *Candida*/bacteria mixed biofilms have still been studied little, especially non-*albicans* *Candida* biofilms.

There have been several studies reporting an *in vitro* effect of growth media, substrates and techniques on biofilms [15–17]. In our previous work [18], we found that different growth media and several culture variables (inoculum concentration, incubation period and feeding conditions) can strongly influence non-*albicans* *Candida* species biofilm formation. However, up to now, only few *in vitro* studies have investigated the mixed species biofilm formation with non-*albicans* *Candida* species and *Staphylococcus*. Therefore, it is important to know the conditions under which mixed biofilms of non-*albicans* *Candida* species and *Staphylococcus* are able to adhere to surfaces of silicone and form biofilms. In this study, we assess the impact of the culture condition factors on mixed species biofilm with non-*albicans* *Candida* species and *Staphylococcus* on silicone *in vitro*.

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2. Material and methods

2.1. Strains and growth media

The strains used in this study were *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis* and *S. epidermidis*, isolated from voice prostheses of laryngectomized patients in routine follow up examinations. The strains were stored in -80°C and thawed before use. Tryptic Soy Broth (TSB) (Sigma-Aldrich, Austria) was utilized for bacteria while yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose [Sigma-Aldrich, Austria]) was used for *Candida* species. Dilutions of TSB, RPMI-1640 (Life Technologies, America) and Brain Heart Infusion Broth (BHI, Sigma-Aldrich, Austria) were used for culturing the mixed biofilms.

2.2. Biofilm formation on medical grade silicone

Biofilms were formed on silicone as described previously [19]. In brief, medical grade silicone plates (diameter: 8 mm, thickness: 3 mm, Websinger, Austria) were steam sterilized at 121°C (20 min) and placed into wells of 96-well microplate. Overnight bacterial and fungal cultures were diluted to a cell density of 1×10^6 cfu/ml in RPMI-1640, TSB or BHI. Equal volumes of bacterial and each of fungal suspensions were mixed. 100 μl diluted mixed microbial suspensions were pipetted into wells of a 96-well microplate. After an adhesion period (90 min) at 37°C , non-attached cells were removed and fresh media was added. The 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 culture medium with or without 10% fetal bovine serum (FBS) was used to evaluate the serum affection on biofilm formation.

2.3. Crystal violet assay

Biofilms were grown and biofilm biomass was evaluated with crystal violet (CV) staining. The plates were washed and stained with 150 μl of 0.1% (w/v) crystal violet for 30 min. The amount of biofilm biomass was determined by measuring its OD₅₉₀.

2.4. 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 [20]. Silicone were washed and incubated for 2 h with 150 μl XTT working reagent (XTT 180 mg/l; AppliChem, Darmstadt, Germany) at 37°C . The resulting absorbance was test with reading at OD₄₉₀.

2.5. Statistical analysis

All the experiments were done in triplicate. Means \pm standard deviations (SD) had been calculated for each experiment. Statistical analysis was performed by analysis of variance (ANOVA) with Tukey post hoc test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Mixed biofilm formation under different growth media

Mixed biofilms out of three *Candida* species, *C. tropicalis*, *C. krusei* and *C. parapsilosis* and *S. epidermidis* were grown in three standard growth media, namely RPMI 1640, BHI and TSB, to evaluate their ability to form biofilms. Results obtained after 24 h (Fig. 1) revealed that mixed biofilm with *C. tropicalis* and *S. epidermidis* exhibited significantly more biofilm formation when grown in BHI medium than the

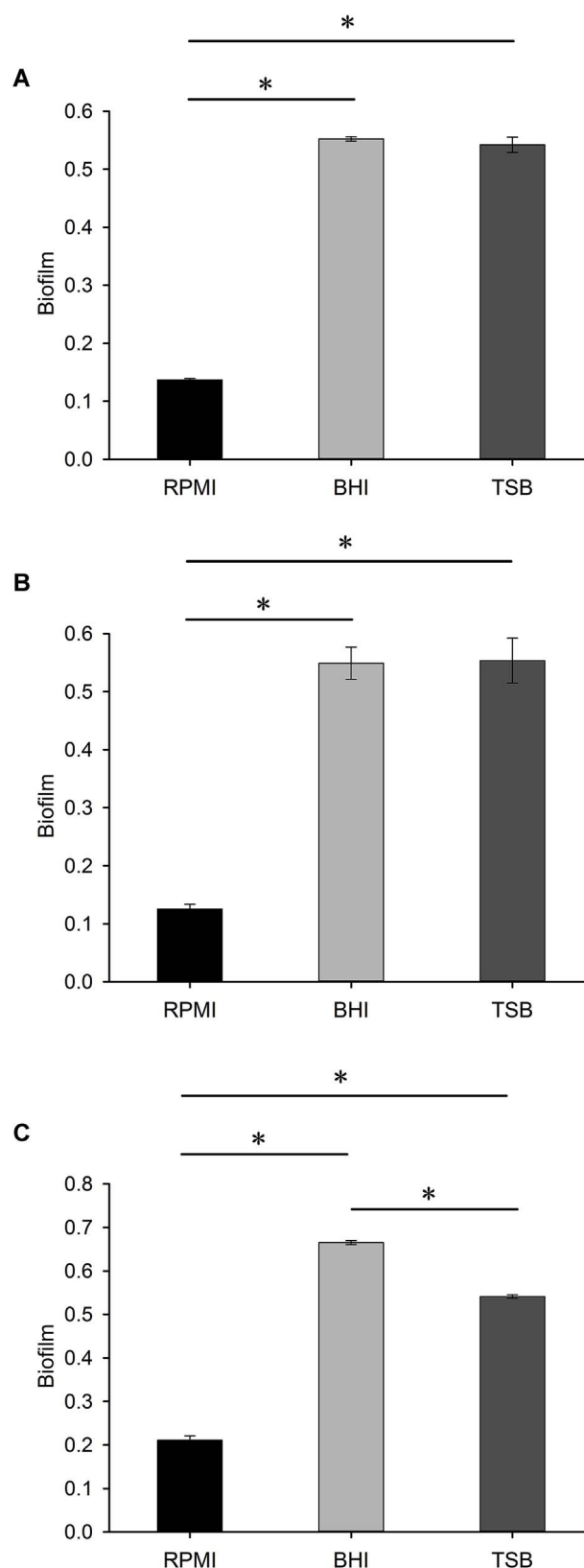


Fig. 1. Biofilm formation by clinically isolated non-albicans *Candida* species and *S. epidermidis* in different media. (A) *C. parapsilosis* and *S. epidermidis*; (B) *C. krusei* and *S. epidermidis*; (C) *C. tropicalis* and *S. epidermidis*. The results shown represent the means and standard deviations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

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