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In vitro modulation of probiotic bacteria on the biofilm of *Candida glabrata*

Shu Yih Chew [a], Yoke Kqueen Cheah [b], Heng Fong Seow [c], Doblin Sandai [d], Leslie Thian Lung Than [a, *]

[a] Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[b] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[c] Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[d] Advanced Medical & Dental Institute, Universiti Sains Malaysia, 13200 Kepala Batas, Pulau Pinang, Malaysia

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ABSTRACT

A conspicuous new concept of pathogens living as the microbial societies in the human host rather than free planktonic cells has raised considerable concerns among scientists and clinicians. Fungal biofilms are communities of cells that possess distinct characteristic such as increased resistance to the immune defence and antimycotic agents in comparison to their planktonic cells counterpart. Therefore, inhibition of the biofilm may represent a new paradigm for antifungal development. In this study, we aim to evaluate the *in vitro* modulation of vulvovaginal candidiasis (VVC)-causing *Candida glabrata* biofilms using probiotic lactobacilli strains. Probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 were shown to have completely inhibited C. *glabrata* biofilms and the results were corroborated by scanning electron microscopy (SEM), which revealed scanty structures of the mixed biofilms of *C. glabrata* and probiotic lactobacilli strains. In addition, biofilm-related *C. glabrata* genes *EPA6* and *YAK1* were downregulated in response to the probiotic lactobacilli challenges. The present study suggested that probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strains inhibited *C. glabrata* biofilm by partially impeding the adherence of yeast cells and the effect might be contributed by the secretory compounds produced by these probiotic lactobacilli strains. Further investigations are required to examine and identify the biofilm inhibitory compounds and the mechanism of probiotic actions of these lactobacilli strains.

1. Introduction

Vulvovaginal candidiasis (VVC) is a common superficial infection of the vaginal mucous membranes caused by the *Candida* species. In fact, VVC affects an estimated 75% of women population at least once in their lifetime [1]. In addition, from 75% of women suffered from VVC, 50% of them are expected to develop a second episode and approximately 5% of them suffered from recurrent, which is defined as having four episodes or more VVC within one year [2]. VVC is predominantly caused by *Candida albicans* (85–90%) but other *Candida* species like *Candida glabrata*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* can also be the etiological agents [3,4]. Unlike VVC in healthy subjects, *C. glabrata* is the most common *Candida* species (39%) isolated from the VVC patients with diabetes mellitus [5]. A large proportion of VVC patients also responded poorly to fluconazole therapy, possibly due to the innate azole resistance of *C. glabrata* [6]. The diagnosis and treatment of VVC, along with the lost productivity have resulted in an estimated cost of US$ 1.8 billion in 1995 and the cost is expected to increase to US$ 3.1 billion by the year 2014 [7].

Biofilm can be defined as a complex three-dimensional microbial structure that comprises of functional community of microorganisms embedded in extracellular matrix [8]. Most of the bacteria and fungi exist primarily in their biofilm form in natural environments [9] and these biofilm communities are believed to cause more than 80% of microbial infections in the human body [10]. In comparison to its planktonic form counterparts, biofilms are more resistant to the onslaught of antimycotic agents [11], which make

* Corresponding author.
E-mail address: tl_leslie@upm.edu.my (L.T.L. Than).

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biofilm infections notoriously difficult to treat and cause frequent relapsing [12]. To date, investigations on the biofilm formation in the pathogenesis of VVC were relatively scarce. Harriott et al. [8] demonstrated that *C. albicans* is able to form biofilms on vaginal epithelium using an *in vivo* murine VVC model. Silva et al. [13] reported that *C. glabrata* originating from the vaginal cavity is the most capable strain in biofilm formation, as compared to those isolated from other body sites. Consequently, in addition to innate azole resistance, high capability of biofilm formation of vaginal *C. glabrata* strains might also strengthen their defence against antimycotic drugs.

A probiotic is defined as "live microorganism which when administrated in adequate amounts confers a health benefit on the host" [14,15]. A randomized clinical trial involving 64 healthy women showed that orally administrated probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 reduced the vaginal colonisation by pathogenic bacteria and yeast [16]. Köhler et al. [17] reported that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were able to suppress fungal growth and even shut down the metabolic activity of the VVC-causing *C. albicans* strain. Furthermore, a randomised, double blind and placebo controlled trial conducted by Martinez et al. [18] reported that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strengthened the efficacy of fluconazole treatment by extending the longevity and effectiveness of this antimycotic agent in VVC patients. Therefore, probiotic bacteria are considered as a new potential preventative and therapeutic agents in women who are suffering from VVC, especially infections caused by non-*C. albicans Candida* (NCAC) species and recurrent attack.

Although *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been proven to be beneficial to women's urogenital health by decreasing the risk of urogenital diseases such as bacteria vaginosis (BV) and VVC [16,18–20], the abilities of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in the inhibition of microbial biofilms have not been studied before. In addition, most of the current investigations focuses on *C. albicans*. There are very few studies done on the biofilms of emerging NCAC species like *C. glabrata*. Hence, the present study was designed to investigate the *in vitro* modulatory effects of probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strains on the biofilm of the human vaginal pathogen *C. glabrata*.

2. Materials and methods

2.1. Microorganisms

Probiotic lactobacilli strains *L. rhamnosus* GR-1 (ATCC 55826) and *L. reuteri* RC-14 (ATCC 55845) were kindly provided by Chr. Hansen A/S (Denmark). *C. glabrata* ATCC 2001 was purchased from the American Type Culture Collection (ATCC, USA). Four vaginal isolates of *C. glabrata* strains, namely *C. glabrata* 91152, *C. glabrata* 94885, *C. glabrata* 95670 and *C. glabrata* 98328 were obtained from University Malaya Medical Centre (UMMC). The identities of the lactobacilli strains were confirmed by a molecular approach using DNA sequencing of the 16S regions with 16S universal primers (P27F and P1492R), while *C. glabrata* strains were confirmed by fungal specific internal transcribed spacer PCR (ITS-PCR) [21].

2.2. Growth media and culture conditions

L. rhamnosus GR-1 and *L. reuteri* RC-14 strains were routinely grown on de Man, Rogosa and Sharpe (MRS) agar (Hi-Media, India) and incubated anaerobically for 48 h at 37 °C. A loopful of bacterial colonies were inoculated into MRS broth (Hi-Media, India), incubated anaerobically for 24 h at 37 °C in an orbital shaker (180 rpm). *C. glabrata* strains including ATCC 2001 and other vaginal isolates were grown on Yeast Extract-Peptone-Dextrose (YPD) agar (Becton Dickinson, USA) and incubated aerobically for 24 h at 37 °C. Similarly, *C. glabrata* colonies were transferred into YPD broth (Becton Dickinson, USA) and incubated aerobically for 24 h at 37 °C in an orbital shaker (180 rpm).

2.3. Preparation of filter-sterilised cell-free supernatant (FCS)

Broth culture of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in MRS broth (Hi-Media, India) were adjusted to OD_{600nm} of 1.0. Subsequently, 2 mL of the broth culture was added into 100 mL of MRS and incubated anaerobically for 48 h at 37 °C (180 rpm) in an anaerobic jar supplemented with AnaeroGen™ 2.5 L Sachet (Oxoid, UK). The cell supernatant was collected following centrifugation at 11,000 × *g* for 10 min and filter-sterilised using sterile 0.22 μM pore-size syringe filter (TPP, Switzerland). The FCS obtained was stored at −20 °C for future use.

2.4. Determination of the lactic acid concentration

A commercial D-lactic acid and L-lactic acid assay kit (Megazyme, Ireland) was used to determine the concentration of lactic acid in the FCS produced by *L. rhamnosus* GR-1 and *L. reuteri* RC-14.

2.5. Biofilm formation

C. glabrata biofilms were prepared on a pre-sterilised, clear and flat bottomed 96-well polystyrene cell culture plates with low-evaporation lids (Becton Dickinson, USA). Briefly, broth cultures of *C. glabrata* ATCC 2001 and four other vaginal isolates were washed twice with phosphate buffered saline (PBS), pH 7.4 and resuspended in Roswell Park Memorial Institute medium (RPMI 1640) without sodium bicarbonate, but supplemented with L-glutamine (Gibco, USA). RPMI 1640 media was buffered with 0.165 M 3-morpholinopropane-1-sulfonic acid, MOPS (Nacalai Tesque, Japan) to pH 7.0. The *C. glabrata* cell suspension was diluted to OD_{600nm} of 0.1 and 100 μL of the cell suspension was dispensed into selected wells in 96-well plate. The 96-well plate was covered with its original lid, sealed with parafilm and incubated for 24 h at 37 °C for biofilm formation.

2.6. XTT reduction assay

The effects of FCS produced by probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 strains on the *C. glabrata* biofilms were determined by using 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. After formation of *C. glabrata* biofilms, cell suspension was aspirated and the biofilm was washed twice with PBS pH 7.4 to remove non-adherent cells. Residual PBS pH 7.4 in the wells were removed and 100 μL of 10% (v/v) and 100% (v/v) of FCS produced by *L. rhamnosus* GR-1 or *L. reuteri* RC-14 were added. In addition, MRS broth acidified with lactic acid (0.3 g/L) was used to determine the effect of lactic acid present in FCS in the biofilm inhibition. The plate was incubated for 24 h at 37 °C. A volume of 100 μL XTT/menadione solution prepared from XTT sodium salt (Sigma–Aldrich, USA) supplemented with menadione (Sigma–Aldrich, USA) at a final concentration of 1 μM was added. The plate was covered in aluminium foil and incubated in the dark for 3 h at 37 °C. Finally, 80 μL of the supernatant were transferred into a new 96-well microtitre plate and the optical density at 490 nm was obtained by using Synergy H1 hybrid multimode microplate reader (Biotek, USA).

2.7. Scanning electron microscopy (SEM)

SEM was employed to visualise the ultrastructure of the