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ORIGINAL ARTICLE

Probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 exhibit strong antifungal effects against vulvovaginal candidiasis-causing *Candida glabrata* isolates

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antifungal, *Candida glabrata*, *Lactobacillus reuteri* RC-14, *Lactobacillus rhamnosus* GR-1, probiotic, vulvovaginal candidiasis.

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Abstract

Aims: This study investigates the antagonistic effects of the probiotic strains *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 against vulvovaginal candidiasis (VVC)-causing *Candida glabrata*.

Methods and Results: Growth inhibitory activities of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains against *C. glabrata* were demonstrated using a spot overlay assay and a plate-based microtitre assay. In addition, these probiotic lactobacilli strains also exhibited potent candidacidal activity against *C. glabrata*, as demonstrated by a LIVE/DEAD yeast viability assay performed using confocal laser scanning microscopy. The metabolic activities of all *C. glabrata* strains were completely shut down in response to the challenges by the probiotic lactobacilli strains. In addition, both probiotic lactobacilli strains exhibited strong autoaggregation and coaggregation phenotypes in the presence of *C. glabrata*, which indicate that these lactobacilli strains may exert their probiotic effects through the formation of aggregates and, thus the consequent prevention of colonization by *C. glabrata*.

Conclusions: Probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains exhibited potent antagonistic activities against all of the tested *C. glabrata* strains. These lactobacilli exhibited antifungal effects, including those attributed to their aggregation abilities, and their presence caused the cessation of growth and eventual cell death of *C. glabrata*.

Significance and Impact of the Study: This is the first study to report on the antagonistic effects of these probiotic lactobacilli strains against the non-*Candida albicans Candida* (NCAC) species *C. glabrata*.

Introduction

Vulvovaginal candidiasis (VVC) is one of the most common gynaecological disorders caused by opportunistic *Candida* species. The treatments employed for an uncomplicated VVC infection caused by *Candida albicans* are usually effective and straightforward because of the broad availability of antimycotic agents. In comparison, a complicated VVC infection, which includes recurrent VVC and VVC caused by non-*C. albicans Candida* (NCAC) species, such as *Candida glabrata*, can be problematic. Numerous antimycotic agents for VVC are widely available in the market place without the need for a prescription from clinicians as over-the-counter (OTC) products (Sobel 1999). However, Ferris *et al.* (2002) have reported that approximately 67% of the self-diagnosed and self-medicated individuals with a presumed VVC who used OTC products were incorrect in the diagnosis of VVC, and instead, the majority of these individuals were infected by bacterial vaginosis or another mixed infection. As a consequence, prolonged and

incorrect self-treatment of VVC using OTC products may lead to the emergence of drug-resistant *Candida* strains (Mathema *et al.* 2001). In fact, both *C. albicans* and *C. glabrata* have been reported to develop cross-resistance towards fluconazole and other OTC drugs such as clotrimazole, miconazole and tioconazole (Cross *et al.* 2000).

The prevalence of NCAC species such as *C. glabrata* increases in patients with recurrent VVC, with up to 20% of the recurrent infections attributed to NCAC species (Ramsay *et al.* 2009). In addition, *C. glabrata* is typically the most common species isolated from the vaginal cavity of a diabetic patient with a VVC infection, and *C. glabrata* has been reported to respond poorly to fluconazole treatment (Goswami *et al.* 2006). The current treatment modalities available for an uncomplicated VVC have been relatively effective. However, in response to the increased prevalence of drug resistant NCAC strains and frequent reoccurrences of infections, new discoveries or 'paradigm shifts' in the therapeutic and preventative approaches for VVC infections are certainly warranted.

Species from the *Lactobacillus* and *Bifidobacterium* genera are generally considered as common inhabitants in the human body that are not detrimental to the human host. In recent years, these benign micro-organisms have gained increasing medical attention primarily because of their antagonistic effects against numerous human pathogens, which makes them a potential therapeutic or prophylactic option for treatments against infectious diseases. To date, an appreciable number of probiotic lactobacilli strains isolated from human origins have been reported to be antagonistic against medically important pathogens. For instance, probiotic lactic acid bacteria have been demonstrated to inhibit the growth of a number of bacterial pathogens, including *Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli* and *Enterococcus faecalis* (Tejero-Sariñena *et al.* 2012).

Investigations of the antifungal activities of probiotic strains are less common than investigations of their antibacterial activities. Rönnqvist *et al.* (2007) reported that a *Lactobacillus fermentum* Ess-1 strain isolated from the human throat exhibited inhibitory activity against growth of both *C. albicans* and *C. glabrata*. In addition, a *Lactobacillus plantarum* 16 strain inhibited the mycelial cells, germ tubes and hyphae of *Aspergillus fumigatus* Af293 (Crowley *et al.* 2013). In addition to *Candida* and *Aspergillus* species, probiotic strains have also been reported to exhibit inhibitory effects against the growth of other fungal pathogens such as *Fusarium* and *Trichophyton* species (Hassan and Bullerman 2008; Guo *et al.* 2011).

The commercially available probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains have been demonstrated to cause significant reductions in vaginal yeast colonization in a randomized clinical trial (Reid *et al.* 2003). In addition, *in vitro* studies of these probiotic strains have also reported that both *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains exhibit inhibitory effects against *C. albicans*, which is the most common *Candida* species that causes VVC (Martinez *et al.* 2009; Köhler *et al.* 2012). To date, the inhibitory effects of probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains have not been tested against the NCAC species *C. glabrata*, which is one of the most common causes of complicated VVC. In addition, the mechanisms that impart the probiotic properties of these lactobacilli strains have yet to be unravelled. Therefore, this study has aimed to investigate the probiotic effects of the *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains against vaginal isolates of the emerging NCAC species *C. glabrata*.

Materials and methods

Micro-organisms

The two probiotic lactobacilli strains *Lact. rhamnosus* GR-1 (ATCC 55826) and *Lact. reuteri* RC-14 (ATCC 55845) were kindly provided by Chr. Hansen A/S (Hørsholm, Denmark). *Candida glabrata* ATCC 2001 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Clinical strains of *C. glabrata* (vaginal isolates), namely *C. glabrata* 91152, *C. glabrata* 94885, *C. glabrata* 95670 and *C. glabrata* 98328, were obtained from the University Malaya Medical Centre (UMMC). The identities of the two lactobacilli strains were confirmed by 16S rDNA sequencing, whereas the fungal specific internal transcribed spacer (ITS) region was used for confirmation of *C. glabrata* strains.

Growth media and culture conditions

Both probiotic lactobacilli strains were routinely cultured on de Man, Rogosa and Sharpe (MRS) agar (Hi-Media, Mumbai, India) and incubated anaerobically for 48 h at 37°C. Subsequently, lactobacilli strains were inoculated into MRS broth (Hi-Media) and incubated anaerobically for 24 h at 37°C in an orbital shaker (180 rev min^{-1}). All of the *C. glabrata* strains were cultured on Yeast Extract-Peptone-Dextrose (YPD) agar (Becton Dickinson, Franklin Lakes, NJ) and incubated aerobically for 24 h at 37°C. *Candida glabrata* colonies were transferred into YPD broth (Becton Dickinson) and incubated aerobically for 24 h at 37°C in an orbital shaker (180 rev min^{-1}).

Spot overlay assay

Primary screening of the growth inhibitory activity of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 against

C. glabrata strains was achieved by conducting spot overlay assays (Köhler *et al.* 2012). Briefly, overnight cultures of *Lact. rhamnosus* GR-1 and *Lact. reuteri RC-14* were diluted to an $OD_{600\ nm}$ of 1·0. Subsequently, 5 μl of a cell dilution was spotted onto MRS agar. Following 48 h of incubation at 37°C under anaerobic conditions, the agar plates with lactobacilli colonies were overlayed with a *C. glabrata* strain resuspended in 0·7% MRS soft agar ($OD_{600\ nm}$ = 0·01). The soft agar was allowed to solidify and the plate was incubated for another 24 h at 37°C. To determine the effect of pH on the growth inhibitory activity of these probiotic strains, lactobacilli dilutions ($OD_{600\ nm}$ = 1·0) were spotted onto MRS-MOPS agar (MRS medium buffered with 0·165 mol l^{-1} 3-morpholinopropane-1-sulphonic, pH 7·0) instead of MRS agar. The size of the clear zones of inhibition of *C. glabrata* growth surrounding lactobacilli colonies were measured after 24 h incubation. Growth inhibition was expressed as the ratio of the diameter of the halo of inhibition (mm)/diameter of the colony (mm).

Preparation of filter-sterilized cell-free supernatant (FCS)

Broth cultures of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 in MRS broth were adjusted to an $OD_{600\ nm}$ of 1·0. Subsequently, 2 ml of the culture were added to 100 ml of MRS and incubated anaerobically in an anaerobic jar supplemented with AnaeroGen™ sachet (Oxoid, Basingstoke, Hampshire, UK). The anaerobic jars were incubated in an orbital shaker (180 rev min^{-1}) for 48 h at 37°C. The cell supernatant was collected following centrifugation at 11 000 ***g*** for 10 min and filter-sterilized using sterile 0·22 μmol l^{-1} pore-size syringe filters (TPP, Trasadingen, Switzerland). The obtained FCS was stored at −20°C.

Plate-based microtitre assay

Secondary screening of the growth inhibitory activity of the FCS produced by *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 against *C. glabrata* strains was conducted using 96-well plate-based microtitre assays. Overnight cultures of *C. glabrata* in YPD broth were diluted to an $OD_{600\ nm}$ of 0·1, and 100 μl of each *C. glabrata* cell dilution was dispensed into 96-well microtitre plate. For a blank control, *C. glabrata* culture was replaced by 100 μl of sterile MRS broth. Aliquots of 100 μl of FCS were added into the designated wells and incubated for 24 h at 37°C. The $OD_{600\ nm}$ was recorded at 2, 4, 6, 8 and 24 h using an MRX microplate reader (Dynex Technologies, Chantilly, VA). To determine the effect of pH on the growth inhibition activity of the FCS, the pH of each FCS was neutralized to pH 7·0 prior to filter-sterilization. The growth inhibition activities of neutralized FCS were tested against all *C. glabrata* strains as well.

Confocal laser scanning microscopy (CLSM)

The candidacidal activities of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 against the *C. glabrata* strains were evaluated by CLSM and by using a commercial LIVE/DEAD® yeast viability kit (Molecular Probes, Eugene, OR) following manufacturer's instructions. Briefly, overnight cultures of lactobacilli and *C. glabrata* strains were diluted to $OD_{600\ nm}$ values of 0·5 and 1·0, respectively. Equal volumes (2 ml) of lactobacilli and *C. glabrata* strains were mixed and co-incubated for 24 h at 37°C. For a nontreated control, *C. glabrata* strains were grown in MRS broth without the inclusion of lactobacilli strains. Cells were washed with GH solution (10 mmol l^{-1} Na-HEPES buffer supplemented with 2% glucose, pH 7·2), mixed with 12·5 μmol l^{-1} FUN-1 cell stain (supplied in the LIVE/DEAD® yeast viability kit) and incubated in the dark for 30 min at 30°C. Subsequently, the cell suspensions were combined with Calcofluor White M2R to a final concentration of 25 μmol l^{-1} and incubated at 30°C in the dark for an additional 10 min. The metabolic activities of the *C. glabrata* cells were observed using a FluoView™ FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) using multipass filter sets appropriate for viewing 4′,6-diamidino-2-phenylindole (DAPI) (350 nm excitation, 470 nm emission), fluorescein (494 nm excitation, 518 nm emission) and rhodamine (580 nm excitation, 605 nm emission). Images were produced using the FV10-ASW VIEWER software, ver. 4.0 (Olympus).

Aggregation assay

The autoaggregation of probiotic lactobacilli strains was determined by a spectrophotometric autoaggregation assay. Briefly, overnight cultures of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains were harvested, washed and diluted to an $OD_{600\ nm}$ of 0·5 in PBS solution (pH 7·4). Subsequently, aliquots of 4 ml of cell suspensions of lactobacilli strains were mixed briefly for 10 s with a vortex mixer and incubated for 4 h or 24 h at 37°C. In addition, the autoaggregation between *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains was also determined. The $OD_{600\ nm}$ values of the cell suspensions were measured using a NanoPhotometer® UV/Vis spectrophotometer, and the percentages of autoaggregation (%) were expressed as $100 \times [1\text{-}(OD_A/OD_B)]$; where, OD_A is the absorbance after 4 or 24 h of incubation and OD_B is the absorbance before incubation.

The levels of coaggregation between the probiotic lactobacilli strains and the *C. glabrata* strains were

determined by a spectrophotometric coaggregation assay. The preparation of the lactobacilli cell suspension was the same as described for the autoaggregation assay, whereas the *C. glabrata* cultures were diluted to an $OD_{600\ nm}$ of 1·0 in PBS solution. A volume of 2 ml of *Lact. rhamnosus* GR-1 or *Lact. reuteri* RC-14 cell suspension was mixed with 2 ml of each *C. glabrata* cell suspension. The cell suspensions were mixed briefly for 10 s and incubated for 4 h at 37°C. The readings were measured as described for the autoaggregation assay. The percentages of coaggregation (%) were expressed as:

$$\text{Percentage of coaggregation}(\%) = 100 \times \frac{[(OD_L + OD_C) - 2(OD_M)]}{(OD_L + OD_C)}$$

where, OD_L is the absorbance of a probiotic lactobacilli strain; OD_C is the absorbance of a *C. glabrata* strain; and OD_M is the absorbance after 4 h of co-incubation.

Microbial adhesion to hydrocarbons (MATH)

The cell surface hydrophobicities of the *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 were determined by a MATH test according to Kos *et al.* (2003) with a slight modification. Xylene and toluene (Nacalai Tesque, Japan) were used as the hydrocarbon solvents in this test. Overnight cultures of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains were prepared as described for the autoaggregation and coaggregation assays ($OD_{600\ nm}$ = 0·5). One millilitre of xylene or toluene was added to 3 ml of each lactobacilli cell suspension, and the solutions were incubated for 10 min at room temperature. Subsequently, the two-phase solutions were vortexed for 2 min and incubated for an additional of 20 min at room temperature. The hydrocarbon was removed completely, and the absorbance of aqueous-phase cell suspension was measured at 600 nm. The percentage of cell surface hydrophobicity (%) was expressed as $100 \times [1\text{-}(OD_A/OD_B)]$; where, OD_A is the absorbance after mixing with hydrocarbon solvents and OD_B is the absorbance before mixing with hydrocarbon solvents.

Statistical analysis

All of the data were expressed as the mean ± SD. Statistical analyses were performed using the GraphPad Prism software ver. 6.0 (GraphPad Software, Inc. La Jolla, CA). The results of autoaggregation and coaggregation assay were subjected to two-way ANOVA tests, followed by Tukey's multiple comparison tests. A *P*-value <0·05 ($P < 0·05$) was considered to be significant.

Results

Growth inhibitory activity of probiotic lactobacilli strains against *Candida glabrata*

The growth inhibitory activity of probiotic lactobacilli strains was demonstrated as both *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains produced visible inhibition zones against all of the tested *C. glabrata* strains. In addition, *C. glabrata* ATCC 2001 was the most sensitive strain to the growth inhibitory effects of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14, with the largest inhibition zones of 1·63 ± 0·04 and 1·54 ± 0·04, respectively (Table 1). To investigate whether pH contributes to the growth inhibitory effects of probiotic lactobacilli strains, the pH of MRS agar was buffered and neutralized by the addition of MOPS to pH 7·0. The probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains still managed to produce visible inhibition zones against all of the tested *C. glabrata* strains despite the buffering of the growth media with MOPS to a pH of 7·0 before inoculation. However, the visible inhibition zones appeared to be smaller than those of the previous experiments that utilized unbuffered MRS agar (Table 1). The growth inhibitory activity of *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 against the tested *C. glabrata* strains was reduced by 23–56% and 20–62%, respectively, when the MRS media were neutralized to pH 7·0.

Secondary screening of the growth inhibitory activities of the FCS produced by the probiotic lactobacilli strains was evaluated using a plate-based microtitre assay. In the nontreated control wells, (in which *C. glabrata* strains were grown in MRS broth), vigorous growth of *C. glabrata* cells was observed ($OD_{600\ nm}$ approx. 1·2–1·5) after 24 h of incubation. However, when *C. glabrata* strains were challenged with the FCS from the probiotic lactobacilli strains, the growth of *C. glabrata* strains was inhibited over the 24 h of incubation time ($OD_{600\ nm}$ approx. 0·3–0·7) (Fig. 1). In concordance with the results from the spot overlay assay, the FCS produced by *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 inhibited the growth of *C. glabrata* ATCC 2001 strain by 73·20 ± 2·26% and by 69·79 ± 5·10%, respectively, which were the highest inhibitory rates observed among all of the tested *C. glabrata* strains (Table 2). The pH neutralization of the FCS produced by the probiotic lactobacilli strains reduced the growth inhibitory activities against *C. glabrata* (Fig. 1), whereby the growth of the *C. glabrata* strains that were challenged by neutralized FCS were only slightly inhibited (approx. 12–20%) compared to the nontreated controls. The neutralized FCS from the probiotic lactobacilli strains only inhibited the growth of the *C. glabrata* strains with a modest efficacy.

Table 1 Ratio of growth inhibition zones of probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains against *Candida glabrata* growth on MRS and MRS-MOPS agar

	C. glabrata strain				
Probiotic strain	ATCC 2001	91152	94885	95670	98328
Lact. rhamnosus GR-1	1·63 ± 0·04	1·33 ± 0·03	1·43 ± 0·14	1·37 ± 0·02	1·43 ± 0·03
Lact. rhamnosus GR-1*	1·48 ± 0·03	1·18 ± 0·04	1·19 ± 0·05	1·16 ± 0·08	1·22 ± 0·06
Lact. reuteri RC-14	1·54 ± 0·05	1·39 ± 0·07	1·52 ± 0·09	1·35 ± 0·06	1·50 ± 0·03
Lact. reuteri RC-14*	1·43 ± 0·03	1·18 ± 0·00	1·20 ± 0·05	1·26 ± 0·07	1·24 ± 0·03

*Cultured in MRS buffered with MOPS (pH 7·0).
The results are expressed as the mean of the ratio of zone inhibition (mm)/the zone of colony growth (mm) obtained from triplicate samples from three independent experiments ± SD.

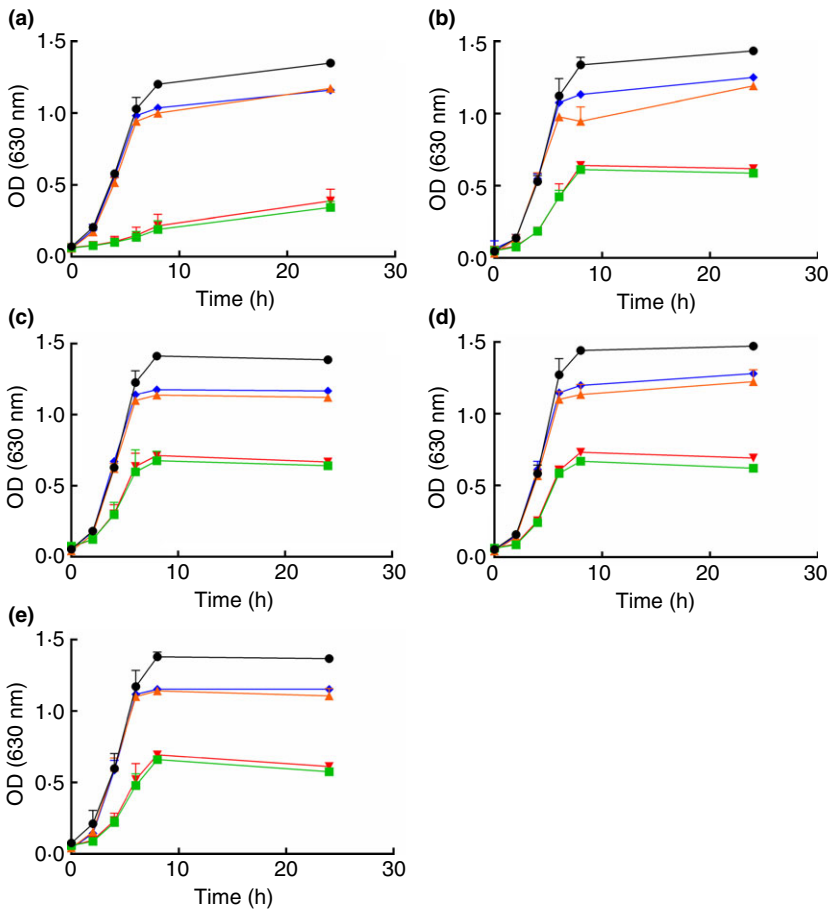

Figure 1 Growth inhibitory activities of FCS and neutralized FCS produced by the probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains against *Candida glabrata*. (a) *C. glabrata* ATCC 2001, (b) *C. glabrata* 91152, (c) *C. glabrata* 94885, (d) *C. glabrata* 95670 and (e) *C. glabrata* 98328. The results are expressed as the mean of triplicate samples of three independent experiments ± SD. (●) Control (MRS), (■) FCS (GR-1), (▲) Neutralized FCS (GR-1), (▼) FCS (RC-14), (◆) Neutralized FCS (RC-14).

Candidacidal effects of the probiotic lactobacilli strains against *Candida glabrata*

In metabolically active fungal cells, green-yellow FUN-1 cell stain is converted into cylindrical intravacuolar structures (CIVS) of an orange-red colour inside the vacuoles of *C. glabrata*. The formation of the CIVS is visible under CLSM observation with the appropriate multipass filter set. In contrast, metabolically inactive or dead fungal cells are incapable of CIVS formation. Thus, viable *C. glabrata* cells with orange-red CIVS can be easily distinguished from dead cells because the dead cells exhibit a diffuse green-yellow fluorescence and lack CIVS. A fungal cell wall-labelling stain, Calcofluor White M2R, was also included for CLSM observation.

As shown in Fig. 2, a monospecies culture of *C. glabrata* ATCC 2001 in MRS broth produced visible orange-red CIVS in the cells when stained by FUN-1 and Calcofluor

Table 2 Percentages of growth inhibitory effects (%) of FCS and neutralized FCS produced by probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains against *Candida glabrata*

	Percentage of growth inhibitory effects (%)			
C. glabrata strain	FCS (GR-1)	Neutralized FCS (GR-1)	FCS (RC-14)	Neutralized FCS (RC-14)
ATCC 2001	73·20 ± 2·26	13·07 ± 0·67	69·79 ± 5·10	14·01 ± 0·67
91152	57·48 ± 1·68	16·84 ± 2·59	55·18 ± 1·87	12·82 ± 1·55
94885	52·89 ± 1·60	19·11 ± 1·03	50·92 ± 2·98	15·79 ± 1·12
95670	56·74 ± 1·05	16·76 ± 5·09	51·85 ± 0·65	12·94 ± 2·11
98328	57·93 ± 0·78	17·96 ± 4·11	55·26 ± 1·70	14·58 ± 2·07

The results are expressed as the mean of triplicate samples from three independent experiments ± SD.

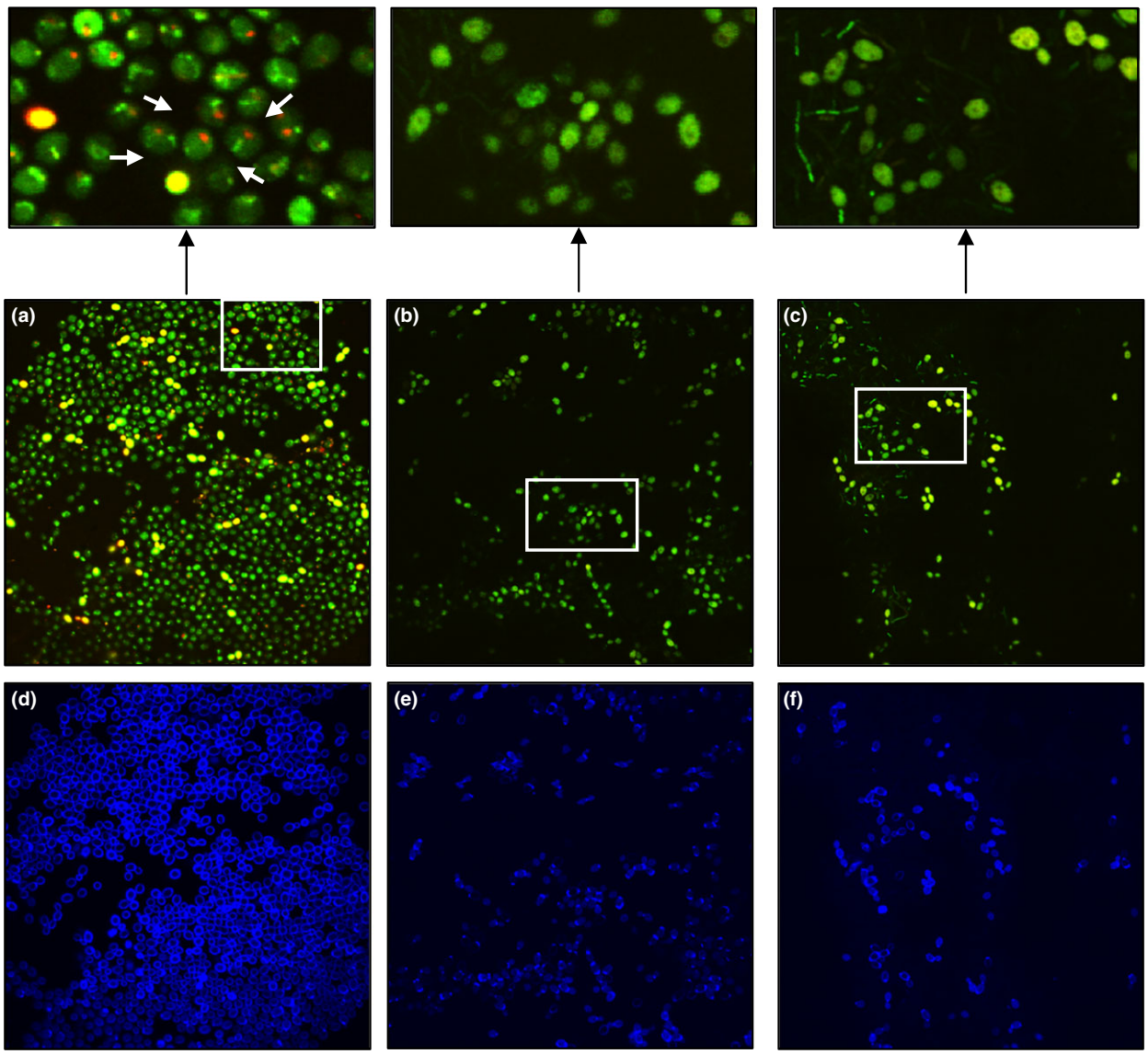

Figure 2 Candidacidal effects of the probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains on the viability of *Candida glabrata* ATCC 2001. CLSM images (magnification: 1000x) showing *C. glabrata* ATCC 2001 stained with FUN-1 and Calcofluor White M2R and viewed using multipass filters for fluorescein and rhodamine (a–c) and DAPI (d–f). (a, d) Monospecies *C. glabrata* ATCC 2001 only; (b, e) *C. glabrata* ATCC 2001 challenged by *Lact. rhamnosus* GR-1 and (c, f) *C. glabrata* ATCC 2001 challenged by *Lact. reuteri* RC-14. The white arrows in the magnified area of the CLSM image indicate formations of orange-red CIVS.

White M2R. This observation indicates that the *C. glabrata* cells were viable and alive because only metabolically active fungal cells are capable of forming CIVS by using an endogenous biochemical processing mechanism. However, the presence of probiotic *Lact. rhamnosus* GR-1 or *Lact. reuteri* RC-14 strains appeared to cause a reduction in the number of metabolically active or viable *C. glabrata* cells. In fact, barely any orange-red CIVS could be detected in *C. glabrata* ATCC 2001 cells that were challenged with the probiotic lactobacilli strains. In addition, the candidacidal effects of the probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains against other vaginal isolates of *C. glabrata* followed similar trends as that against *C. glabrata* ATCC 2001; almost all of the *C. glabrata* cells appeared as diffuse green-yellow fluorescence following the challenge with probiotic lactobacilli (data not shown).

Autoaggregation, coaggregation and cell surface hydrophobicity of probiotic lactobacilli strains

The percentages of autoaggregation were measured and calculated after the incubation of the probiotic lactobacilli strains for 4 and 24 h. Both probiotic lactobacilli strains exhibited a strong autoaggregation phenotype. *Lactobacillus reuteri* RC-14 proved to have a stronger capability to form autoaggregates and exhibited significantly higher autoaggregation rates at both the 4 and 24 h incubation times (31·44 ± 2·30% and 67·80 ± 1·08%, respectively) compared to *Lactobacillus rhamnosus* GR-1 and compared to the autoaggregation between *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 (Table 3).

The coaggregation of each *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strain against the *C. glabrata* strains was evaluated by a spectrophotometric coaggregation assay. Both probiotic lactobacilli strains exhibited a substantial degree of coaggregation against all of the *C. glabrata* strains. Similar to the results obtained from the autoaggregation assay, *Lact. reuteri* RC-14 exhibited a significantly higher percentage of coaggregation against all the tested *C. glabrata* strains compared to *Lact. rhamnosus* GR-1 (Table 4).

The cell surface hydrophobicity of the probiotic strains has been suggested to be associated with the coaggregation abilities of strains. Therefore, the MATH test was used to evaluate the cell surface hydrophobicity of both the *Lact. rhamnosus* GR-1 and the *Lact. reuteri* RC-14 probiotic strains by measuring their absorption to two different hydrocarbons, xylene and toluene. The absorption of the *Lact. reuteri* RC-14 strain to each hydrocarbon was >90%; meanwhile, the absorption values of the *Lact. rhamnosus* GR-1 strain to each hydrocarbon were in the range 10–20% (Table 5). These results indicate that *Lact. rhamnosus* GR-1 is relatively hydrophilic, whereas *Lact. reuteri* RC-14 appeared to be a hydrophobic strain. The results obtained from MATH tests were in concordance with the results from the coaggregation assay, and the higher coaggregation ability of *Lact. reuteri* RC-14 might be partially attributed to the hydrophobic nature of this strain. Nevertheless, other factors such as adhesins might also affect the coaggregation properties of lactobacilli strains because the hydrophilic *Lact. rhamnosus* GR-1 strain still exhibited substantial coaggregation against *C. glabrata*.

Table 3 Percentages of autoaggregation (%) of probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains

	Percentage of autoaggregation (%)	
Probiotic strain	4 h	24 h
Lact. rhamnosus GR-1	21·23 ± 5·31[a]	60·31 ± 2·58[a]
Lact. reuteri RC-14	31·44 ± 2·30[b]	67·80 ± 1·08[b]
Lact. rhamnosus GR-1 + *Lact. reuteri* RC-14	23·75 ± 2·30[a]	65·19 ± 2·18[a,b]

The results are the mean of triplicate samples from three independent experiments ± SD. Different letters indicate statistically significant differences among rows within a column ($P < 0·05$).

Table 4 Percentages of coaggregation (%) of probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains against *Candida glabrata*

	Percentage of coaggregation (%)	
C. glabrata strain	*Lact. rhamnosus* GR-1	*Lact. reuteri* RC-14
ATCC 2001	57·37 ± 3·23[a]	68·40 ± 2·16[b]
91152	61·15 ± 1·21[a]	71·06 ± 3·01[b]
94885	60·29 ± 1·88[a]	70·44 ± 0·34[b]
95670	58·45 ± 3·00[a]	67·44 ± 0·91[b]
98328	58·57 ± 1·15[a]	66·81 ± 5·04[b]

The results are the mean of triplicate samples of three independent experiments ± SD. Different letters indicate statistically significant differences between columns ($P < 0·05$).

Table 5 Cell surface hydrophobicity (%) of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains as determined by a MATH test

	Cell surface hydrophobicity (%)	
Probiotic strain	Xylene	Toluene
Lact. rhamnosus GR-1	17·55 ± 5·94	11·57 ± 2·93
Lact. reuteri RC-14	94·22 ± 1·21	94·05 ± 4·06

The results are expressed as the mean of triplicate samples of three independent experiments ± SD.

Discussion

The probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains inhibited the growth of *C. glabrata* ATCC 2001 and other vaginal isolates of *C. glabrata*. Clear visible inhibition zones were observed around the probiotic lactobacilli strains. The growth inhibitory effects of probiotic lactobacilli strains might be partially attributed to the low pH and the production of organic acids. On the MRS agar buffered with MOPS (neutral pH), the inhibition zones were smaller than those of the unbuffered MRS agar (low pH), suggesting an inverse relationship between pH and *C. glabrata* growth.

However, the pH neutralization of the MRS agar did not completely diminish the growth inhibitory effects of either probiotic lactobacilli strain. This observation suggests that other inhibitory mechanisms or pathways could play roles in the inhibitory effects on *C. glabrata* as well. Apart from the production of organic acids such as lactic acid (De Keersmaecker *et al.* 2006), other mechanisms such as competition for nutrients (Sonnenburg *et al.* 2006) and the production of antimicrobial substances such as bacteriocins (Cleusix *et al.* 2007), biosurfactants (Gudiña *et al.* 2010) and H_2O_2 (Pridmore *et al.* 2008) have been suggested to have contributed to the antagonistic effects of probiotic lactobacilli strains against a variety of pathogens.

In contrast with the *Lact. rhamnosus* GR-1 strain, which is a non-H_2O_2 producer, the probiotic *Lact. reuteri* RC-14 strain is capable of producing H_2O_2 and the potent bacteriocin 3-HPA (Talarico and Dobrogosz 1989). Because the present study did not show that *Lact. reuteri* RC-14 is more potent at inhibiting the growth of *C. glabrata* compared to *Lact. rhamnosus* GR-1, the growth inhibitory effects observed were not likely to be attributed to the production of H_2O_2. In addition, *C. glabrata* was reported to exhibit a higher resistance towards reactive oxygen species (ROS) such as H_2O_2 than to *Saccharomyces cerevisiae* and *C. albicans* (Cuéllar-Cruz *et al.* 2008), which decreases the likelihood of the involvement of H_2O_2 in the observed growth inhibitory effects. Overall, the presence of lactic acid and the low pH level likely play the most prominent roles in imparting the growth inhibitory effects of these probiotic lactobacilli strains. Nevertheless, the involvement of other antimicrobial substances cannot be ruled out because the probiotic lactobacilli strains still exerted a moderate growth inhibitory effect on *C. glabrata* after pH neutralization.

The fungicidal or candidacidal effects of these probiotic lactobacilli strains were evaluated (with fluorescence probes) using a LIVE/DEAD® yeast viability kit and CLSM. The determination of cell viability by colony counting is time consuming and the results usually do not report on the metabolic activity of slow-growing or nondividing cells (Millard *et al.* 1997). In addition, although a technique using the fluorogenic stains calcein acetoxymethyl ester and ethidium homodimer-1 is highly effective for the staining of mammalian cells to differentiate viable and dead cells, it is not applicable to yeast cells because there is inconsistency in the stain permeability and nonspecific surface labelling can occur when these fluorogenic stains are used for yeast cells (Kaneshiro *et al.* 1993). In this study, the FUN-1 stain was chosen because it is a highly sensitive indicator that generates fluorescence patterns that can be used to differentiate yeast cell viability. In addition, the stain has been demonstrated to work effectively in *S. cerevisiae* and several other species of yeast and fungi.

In fact, the FUN-1 stain exploits an endogenous biochemical processing mechanism that appears to be conserved in numerous fungi (Millard *et al.* 1997). Therefore, only metabolically active yeast cells with intact plasma membranes will be able to convert the green fluorescent FUN-1 stain into orange-red fluorescent CIVS. As a consequence, viable and dead yeast cells can be differentiated because viable cells are clearly marked by CIVS, whereas dead yeast cells exhibit a diffuse green-yellow fluorescence. In the CLSM observations, the formation of orange-red fluorescent CIVS was only detected in the monospecies *C. glabrata* cultures that did not contain a probiotic lactobacilli strain. As expected, the presence of the probiotic lactobacilli strains appeared to completely inhibit the metabolic activity of *C. glabrata*; no orange-red fluorescent CIVS formation was observed and the *C. glabrata* cells appeared to become a diffuse green-yellow fluorescence following the challenges from the probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains. Fayol-Messaoudi *et al.* (2005) demonstrated that a nonlactic acid antimicrobial compound produced by *Lact. rhamnosus* GR-1 drastically reduced the viability of *Salm.* Typhimurium. In addition, the candidacidal activity might be attributed to a heat labile, nonlactic acid antimicrobial compound produced by *Lact. rhamnosus* GR-1 (molecular weight >12–14 kDa) that has been identified by McGroarty and Reid (1988). However, the actual candidacidal mechanisms of these probiotic lactobacilli strains still remain to be determined.

The formation of multicellular lactobacilli aggregates is believed to be crucial for the colonization of mucosal surfaces such as those of the oral and urogenital cavities (Reid *et al.* 1990). The aggregation properties of lactobacilli are divided into autoaggregation, which is demonstrated by the formation of a clump (aggregate) of the lactobacilli strain only, and coaggregation, which is characterized by formation of aggregates between lactobacilli

and other genetically distinct cells such as bacterial or fungal pathogens (Ekmekci *et al.* 2009). The ability to adhere on the mucosal surface of epithelial cells is regarded as one of the most important criteria for probiotic selection (Kos *et al.* 2003). In addition, the ability to autoaggregate appears to be required for the adhesion of epithelial cells (Del Ras *et al.* 2000). In this study, the autoaggregation capabilities of the probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains were assessed by a spectrophotometric assay. The results demonstrated that both lactobacilli strains exhibit a strong autoaggregation phenotype. In fact, the autoaggregation capabilities of these lactobacilli strains were time-dependent, and the activity of the *Lact. reuteri* RC-14 strain appeared to be greater than that of *Lact. rhamnosus* GR-1. Coaggregation with other pathogenic micro-organisms is one of the most recognized probiotic mechanisms of lactobacilli strains. Lactobacilli can exert their probiotic effects by creating a hostile niche for the pathogens through the formation of coaggregates and thus prevent colonization by pathogenic micro-organisms (Younes *et al.* 2012). In this study, both the probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains exhibited strong coaggregation activities against *C. glabrata* strains. Furthermore, for all of the *C. glabrata* strains tested, the *Lact. reuteri* RC-14 strain was the superior strain in terms of the formation of coaggregates.

The MATH test first described by Rosenberg *et al.* (1980) is a reliable method that has been extensively used to measure the cell surface hydrophobicity of probiotic lactobacilli strains (Kos *et al.* 2003; Ekmekci *et al.* 2009). The physiochemical properties of lactobacilli, such as their cell surface hydrophobicity, have been suggested to potentially play a prominent role in the autoaggregation and coaggregation of cells (Colloca *et al.* 2000). According to Colloca *et al.* (2000), the cell surface hydrophobicity of lactobacilli strain can be grouped into low hydrophobicity or hydrophilic (0–35%), moderate hydrophobicity (36–70%) and high hydrophobicity (71–100%). In the present study, *Lact. reuteri* RC-14 was characterized by a high hydrophobicity (>90%) after a 4 h co-incubation with xylene and toluene. The results confirmed that *Lact. reuteri* RC-14 is indeed a hydrophobic probiotic strain, whereas *Lact. rhamnosus* GR-1 is a hydrophilic strain (Reid *et al.* 1992).

In addition, the level of cell surface hydrophobicity of probiotic lactobacilli strains correlated well with their autoaggregation and coaggregation abilities. The formation of aggregates was relatively enhanced in the high hydrophobicity strain (*Lact. reuteri* RC-14), whereas the low hydrophobicity (hydrophilic) strain exhibited a relatively reduced aggregation capability (*Lact. rhamnosus* GR-1). The variations in the nature of cell surface components could account for the observed differences in cell hydrophobicity of these two probiotic lactobacilli strains. Numerous studies of the cell surface physiochemistry of micro-organisms have revealed that high hydrophobicity is likely to be attributed to a (glycol-) proteinaceous compound present on cell surface (Cuperus *et al.* 1995; Pelletier *et al.* 1997). In contrast, low hydrophobicity is primarily associated with the presence of polysaccharides on the cell surface.

In conclusion, the present study demonstrated the antagonistic effects of the probiotic *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 strains against an NCAC species, the vaginal pathogen *C. glabrata*. These probiotic lactobacilli strains were shown to impede the growth of and completely inhibit the metabolic activity of *C. glabrata*, which suggests that *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 might be fungicidal to *C. glabrata*. In addition, the strong autoaggregation and coaggregation phenotypes observed in these lactobacilli strains appear to be an important mechanism to antagonize *C. glabrata*. Therefore, *Lact. rhamnosus* GR-1 and *Lact. reuteri* RC-14 may represent a potential alternative option for the treatment of complicated VVC infections caused by *C. glabrata*.

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Conflict of interest

There are no conflicts of interest to declare.

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