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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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 toconazole (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 microorganisms 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-Sarriñena et al. 2012).

Investigations of the antifungal activities of probiotic strains are less common than investigations of their anti-bacterial 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
Lactobacilli inhibit C. glabrata  

S.Y. Chew et al.

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-morpholinepropane-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 µm 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 inhibitory 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 × [1-(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:

$$\text{Percentage of coaggregation(%) } = 100 \times \frac{[(\text{OD}_L + \text{OD}_C) - 2(\text{OD}_M)]}{(\text{OD}_L + \text{OD}_C)}$$

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 $100 \times [1-(\text{OD}_A/\text{OD}_B)]$; 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.
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 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 | 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 | 1.9 ± 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).
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

| 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; (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

| Probiotic strain | Percentage of autoaggregation (%) |
|------------------|----------------------------------|
|                  | 4 h                              |
|                  | 21.23 ± 5.31<sup>a</sup>          |
| Lact. rhamnosus GR-1 | 60.31 ± 2.58<sup>a</sup>          |
| Lact. reuteri RC-14 | 31.44 ± 2.30<sup>b</sup>          |
| Lact. rhamnosus GR-1 + Lact. reuteri RC-14 | 23.75 ± 2.30<sup>b</sup>          |

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

| C. glabrata strain | Lact. rhamnosus GR-1 | Lact. reuteri RC-14 |
|--------------------|----------------------|---------------------|
| ATCC 2001          | 57.37 ± 3.23<sup>a</sup> | 68.40 ± 2.16<sup>b</sup> |
| 91152              | 61.15 ± 1.21<sup>a</sup> | 71.06 ± 3.01<sup>b</sup> |
| 94885              | 60.29 ± 1.88<sup>a</sup> | 70.44 ± 0.34<sup>b</sup> |
| 95670              | 58.45 ± 3.00<sup>a</sup> | 67.44 ± 0.91<sup>b</sup> |
| 98328              | 58.57 ± 1.15<sup>a</sup> | 66.81 ± 5.04<sup>b</sup> |

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

| Probiotic strain | Cell surface hydrophobicity (%) |
|------------------|--------------------------------|
| Lact. rhamnosus GR-1 | Xylene: 17.55 ± 5.94 Toluene: 11.57 ± 2.93 |
| Lact. reuteri RC-14 | Xylene: 94.22 ± 1.21 Toluene: 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 (Gudina et al. 2010) and H$_2$O$_2$ (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$_2$O$_2$ producer, the probiotic *Lact. reuteri* RC-14 strain is capable of producing H$_2$O$_2$ and the potent bacteriocin 3-HPA (Talarico and Dobrogosz 2008), which decreases the likelihood of the involvement of 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 several other species of yeast and fungi.
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 \textit{Lact. rhamnosus} GR-1 and \textit{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 \textit{Lact. reuteri} RC-14 strain appeared to be greater than that of \textit{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 \textit{Lact. rhamnosus} GR-1 and \textit{Lact. reuteri} RC-14 strains exhibited strong coaggregation activities against \textit{C. glabrata} strains. Furthermore, for all of the \textit{C. glabrata} strains tested, the \textit{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, \textit{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 \textit{Lact. reuteri} RC-14 is indeed a hydrophobic probiotic strain, whereas \textit{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 (\textit{Lact. reuteri} RC-14), whereas the low hydrophobicity (hydrophilic) strain exhibited a relatively reduced aggregation capability (\textit{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 (Cupe-rus 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 \textit{Lact. rhamnosus} GR-1 and \textit{Lact. reuteri} RC-14 strains against an NCAC species, the vaginal pathogen \textit{C. glabrata}. These probiotic lactoba-cilli strains were shown to impede the growth of and completely inhibit the metabolic activity of \textit{C. glabrata}, which suggests that \textit{Lact. rhamnosus} GR-1 and \textit{Lact. reuteri} RC-14 might be fungicidal to \textit{C. glabrata}. In addition, the strong autoaggregation and coaggregation phenotypes observed in these lactobacilli strains appear to be an important mechanism to antagonize \textit{C. glabrata}. Therefore, \textit{Lact. rhamnosus} GR-1 and \textit{Lact. reuteri} RC-14 may represent a potential alternative option for the treatment of complicated VVC infections caused by \textit{C. glabrata}. Acknowledgements We acknowledge Chr. Hansen A/S and Prof. Dr. Ng Kee Peng for their kind gifts of the probiotic \textit{Lact. rhamnosus} GR-1 and \textit{Lact. reuteri} RC-14 strains and the vaginal isolates of \textit{C. glabrata} strains that enabled this work. This work was financially supported by RUGS grant (04-01-12-1609RU) from Universiti Putra Malaysia (UPM). Conflict of interest There are no conflicts of interest to declare. References Cleusix, V., Lacroix, C., Vollenweider, S., Duboux, M. and Le Blay, G. (2007) Inhibitory activity spectrum of reuterin produced by \textit{Lactobacillus reuteri} against intestinal bacteria. \textit{BMC Microbiol} \textbf{12}, 101. Colloca, M.E., Ahumada, M.C., López, M.E. and Nader-Macías, M.E. (2000) Surface properties of lactobacilli isolated from healthy subjects. \textit{Oral Dis} \textbf{6}, 227–233. Cross, E.W., Park, S. and Perlin, D.S. 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