Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

In vitro inhibitory activity of probiotic products against oral *Candida* species

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Keywords
antifungal agents, *Bacillus subtilis*, *Candida spp.*, candidiasis, probiotics.

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2015/2504: received 3 December 2015, revised 19 February 2016 and accepted 7 March 2016

doi:10.1111/jam.13138

Abstract

Aims: To evaluate the inhibitory activity of probiotics against oral *Candida* species.
Methods and Results: Four commercial probiotic products were screened. *Bacillus subtilis* R0179 was found to have a significant antifungal effect. *Bacillus subtilis–Candida* interactions were evaluated using disc diffusion tests, confocal laser scanning microscopy, scanning electron microscopy and interaction with engineered human oral mucosa tissue. *Bacillus subtilis* exhibited clear zones of inhibition for *Candida albicans* and *Candida parapsilosis* but not for *Candida krusei*. A remarkable reduction in the number of *Candida* cells and abundant *Candida* cell death were visualized with confocal laser scanning microscopy. Shrinkage and deformation of *Candida* cells was observed using scanning electron microscopy. Culture of *C. albicans* on engineered human oral mucosa tissues resulted in the presence of a large number of yeast cells on the tissue surface and the development of large-scale tissue damage. However, comparatively fewer *Candida* cells were observed on *B. subtilis*-treated tissues. We also use ultra performance liquid chromatography/time of flight mass spectrometry (UPLC/TOF MS) to explore the preliminary antifungal mechanism of *B. subtilis* R0179 and to detect that whether it can secrete an antifungal agent, Iturin A.
Conclusions: *Bacillus subtilis* R0179 exhibits a significant inhibitory effect on the growth of *Candida* species.
Significance and Impact of the Study: *Bacillus subtilis* has the potential to be used in the prevention or treatment of oral candidiasis.

Introduction

Probiotics are micro-organisms that have been claimed to provide health benefits when consumed. In the last two decades, many studies have focused on their roles and effects on the maintenance of health (Lee and Kim 2014). Current researches have shown that the balance between beneficial and pathogenic bacteria is essential in order to maintain health (Bizzini *et al.* 2012). Probiotics confer health benefits on the host via diverse mechanisms, including preventing pathogen adherence, producing bacteriocins, changing the pH, producing vitamins and immunological modulation (Hanson *et al.* 2014).

Probiotics can be applied to plant diseases, animal diseases and human diseases. Most of the clinical interest in probiotics has been focused on their use in the prevention or treatment of gastrointestinal diseases. Probiotics can reduce the duration of acute diarrhoea and improve inflammatory bowel disease symptomatology (Verna and Lucak 2010). Probiotics can also be applied to treat conditions affecting the respiratory tract, urinary tract, skin and vagina (Vandenplas *et al.* 2015). There is an extremely diverse range of potential biological effects, and new functional activities are constantly being explored. Probiotic bacteria have proven beneficial for a number of health conditions including allergic diseases, metabolic

syndrome and infectious diseases (Rijkers *et al.* 2010). Some infectious diseases cannot be cured by antibiotics but can be cured by beneficial microbes. For example, the *Clostridium difficile* infection can be caused by prolonged use of antibiotics, so it is very difficult to treat it using antibiotics (Bakken *et al.* 2011). However, it can be cured by beneficial microbes (Van Nood *et al.* 2013). Infusion of health donor faeces into the colon can correct the imbalance and enable normal bowel function in patients with the *Cl. difficile* infection, because faeces from health donors can re-establish the wide diversity of the intestinal flora (Brandt *et al.* 2012; Saha *et al.* 2013).

The human oral cavity, gastrointestinal tract and vagina have a lot in common (Tlaskalova-Hogenova *et al.* 2004). They all contact with the outside environment and harbour micro-organisms (Relman and Falkow 2001). It has been reported that most vaginal bacteria originate from intestinal microbes (Hanson *et al.* 2014). Oral bacteria and intestinal bacteria are also linked by saliva (Saha *et al.* 2012). The oral cavity can also share flora with the vagina. A study shows that the treatment of the male sexual partner's *Candida* colonization (oral cavity, penile coronal sulcus, seminal fluid) seems to be important in the prevention of recurrent vulvovaginitis (Spinillo *et al.* 1992).

Over the last few years, it has also been suggested that probiotics could be used to maintain oral health. Probiotics have been shown to modulate the oral microbiota, specifically inhibiting the growth of pathogens such as *Streptococcus mutans*, a cariogenic bacterium and *Candida albicans* (*C. albicans*), an organism responsible for the initiation and progression of oral candidiasis (Saha *et al.* 2013). Oral candidiasis is a common opportunistic fungal infection caused by *Candida* species, most commonly *C. albicans* (Coco *et al.* 2008). *Candida albicans* can also cause intestinal candidiasis and vulvovaginal candidiasis. In the last two decades, *C. albicans* has been given much more attention due to the increased incidence with the rise in the number of immunocompromised patients following the AIDS epidemic, overuse and misuse of broad-spectrum antibiotics and aggressive anticancer therapy (Cutler *et al.* 2007). Nowadays due to the rapid development of resistance to common antifungal drugs (Hoehamer *et al.* 2010), such as fluconazole (Lopez *et al.* 2001), new strategies are urgently needed to prevent and treat infections caused by these pathogens.

A few studies have focused on using probiotics to prevent infections caused by *C. albicans* in the oral cavity (Ahola *et al.* 2002; Hatakka *et al.* 2007; Sutula *et al.* 2012). In a previous study, the consumption of cheese that contains the probiotics *Lactobacillus GG*, *Lactobacillus rhamnosus LC705* and *Propionibacterium freudenreichii* ssp. *shermanii JS* was shown to be an effective means of controlling oral *Candida* and hypo-salivation in the elderly (Hatakka *et al.* 2007). Additionally, cheese containing *Lactobacillus GG* and *Lact. rhamnosus LC705* reduced the level of salivary yeasts in healthy adults (Ahola *et al.* 2002). However, the effectiveness of probiotics for oral candidiasis remains controversial. For example, it was reported that consumption of a probiotic drink containing *Lactobacillus casei* strain Shirota had no overall effect on selected oral parameters in healthy denture wearers (Sutula *et al.* 2012). Researches into the use of probiotics to treat oral candidiasis are very limited and the actual antifungal mechanism of probiotics remains unclear. Therefore, further investigations are required to investigate the effectiveness of using probiotics to treat oral *Candida* species, and the mechanism by which this occurs.

The aim of our study is to evaluate the *in vitro* inhibitory activity of four commercial probiotic products against oral *Candida* species and to investigate the possible anti-*Candida* mechanisms. The ultimate goal of the study is to indicate potential applications of probiotic products for oral candidiasis.

Materials and methods

Probiotic products and microbial culture

Four probiotic products were cultured on various agar media. Entrocoordinatibiogen (Shenyang NO. 1 Pharm. Co., Ltd., Shenyang, Liaoning, China), which contains *Bacillus licheniformis*, was propagated on Luria-Bertani (LB) agar (02-137; AOBOX Biotechnology, Beijing, China). Medilac-Vita (Hanmi Pharm. Co., Ltd., Beijing, China), containing *Bacillus subtilis* and *Enterococcus faecium*, was propagated on LB agar. Golden Bifidobacterium tablets (Inner Mongolia ShuangQi Pharmaceutical Co., Ltd., Hohhot, Inner Mongolia, China), containing *Bifidobacterium*, *Lactobacillus bulgaricus* and *Streptococcus thermophiles* was propagated on de Man, Rogosa and Sharpe (MRS) agar (02-291; AOBOXing Product). Bifid Triple Viable (Shanghai Pharmaceuticals Holding Co., Ltd., Shanghai, China), containing *Bifidobacterium*, *Lactobacillus acidophilus* and *Enterococcus faecalis*, was propagated on MRS agar.

Candida strains and susceptibility testing

Five *C. albicans* clinically isolated from oral candidiasis patients were studied as well as four standard strains, *C. albicans* ATCC 90028, *C. albicans* SC5314, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. All the *Candida* strains were propagated on Sabouraud Dextrose Agar (SDA) (02-359; AOBOXing Product) at 37°C. Disc diffusion testing of the four probiotic prod-

ucts was performed as described in the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards (NCCLS)) document M44-A, with minor modifications. Specifically, SDA was used instead of Mueller-Hinton agar. The agar surface was inoculated using a swab dipped in a suspension of *Candida* species adjusted to the turbidity of a 0·5 McFarland standard, and the plates were dried at room temperature for 15 min before placing the blank discs on the surface. Different probiotics (12·5 μl of centrifuged deposit per disc, 3×10^{10} CFU ml^{-1}) and fluconazole (Flz, 12·5 μl per disc, 25 μg as quality control) discs were placed onto the surface of the inoculated plates. The plates were incubated at 37°C for 48 h, at which point the inhibition diameters around the discs were measured. The experiments were performed in triplicate.

Confocal laser scanning microscopy

The suppressive effect of *B. subtilis* on *C. albicans* biofilm formation was confirmed by confocal laser scanning microscopy (CLSM) and Live/Dead staining. Biofilms on plastic coverslips stained using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA). Using this assay, cell viability was determined, as live cells were stained fluorescent green; while those with damaged membranes were stained fluorescent red (Rene *et al.* 2014). Each plastic coverslip was immersed in 200 μl of the Live/Dead BacLight Bacterial Viability reagent and incubated for 20 min in the dark at 30°C. Afterwards, the stained samples were viewed using a CLSM system equipped with two lasers (488 and 543 nm). We also verified the viable and nonviable yeast cells by visual inspection according to their distinct fluorescent emissions. The experiments were performed three times.

Scanning electron microscopy

Visualization of morphological changes of *C. albicans* was achieved using scanning electron microscopy (SEM). Samples were fixed and dehydrated as described previously (Chandra *et al.* 2008). The stubs were sputter-coated with Au/Pd (60/40) for 60s using a sputter-coating machine. Next, the stubs were transferred to the sample-holding platform in the scanning electron microscope. The specimen surface was scanned and representative areas were selected and recorded.

Interaction of *Candida albicans* with engineered human oral mucosa

Normal human gingival cells (fibroblasts) and the HaCaT cell line (epithelial cells) were obtained from the central laboratory at the Peking University School of Stomatology. The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). The epithelial cells were cultured in Dulbecco's modified Eagle's (DME's)—Ham's F12, with 10% FBS medium. The engineered human oral mucosa (EHOM) model was set up as described previously (Rouabhia and Deslauriers 2002). *Candida albicans* (200 μl; 1×10^6 CFU ml^{-1}), with or without *B. subtilis* R0179 (1×10^9 CFU ml^{-1}), were overlaid on the top of the EHOM and co-cultured at 37°C for 24 h. The samples were fixed and periodic acid-Schiff (PAS) staining was performed.

UPLC/TOF MS

The *B. subtilis* R0179 strains were activated in LB plates at 37°C for 24 h. The seed cultures were cultivated in 40 ml LB medium by 10% amount of inoculum at 30°C, under 150 rev min^{-1} for 2 days. The extraction and measurement of Iturin A was carried out as previously reported (Hsieh *et al.* 2008). Putative lipopeptide was analysed by means of UPLC/TOF MS, using the following solvent gradient for chromatography (0·4 ml min^{-1}, 45°C): starting with a mixture (10/90, v/v) of water and methanol, the methanol content was increased to 100% within 8 min, and then kept constant for 1 min.

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM 5 (GraphPad Software, San Diego, CA). The total number of CFU and the percentage of *C. albicans* death were compared using Student's *t*-test. *P* values of <0.05 were considered to indicate significance.

Results

Anti-*Candida* activity by disc diffusion testing

Among the four probiotic products, only Medilac-Vita exhibited a clear inhibition zone against *C. albicans*, while Entrocoordinatibiogen, Golden Bifidobacterium tablets and Bifid Triple Viable did not display inhibition activity in the disc diffusion assay upon screening. Subsequently, we isolated and tested the bacteria components, *B. subtilis* and *Ent. faecium*, respectively, from Medilac-Vita. It transpired that *B. subtilis* exhibited rather strong anti-*Candida* activity with clear zones of inhibition against *C. albicans* ATCC 90028 (Fig. 1a), *C. albicans* SC5314 (Fig. 1b) and *C. parapsilosis* ATCC 22019 (Fig. 1d), although it did not exhibit notable inhibition against *C. krusei* ATCC 6258 growth (Fig. 1c). The diameters of

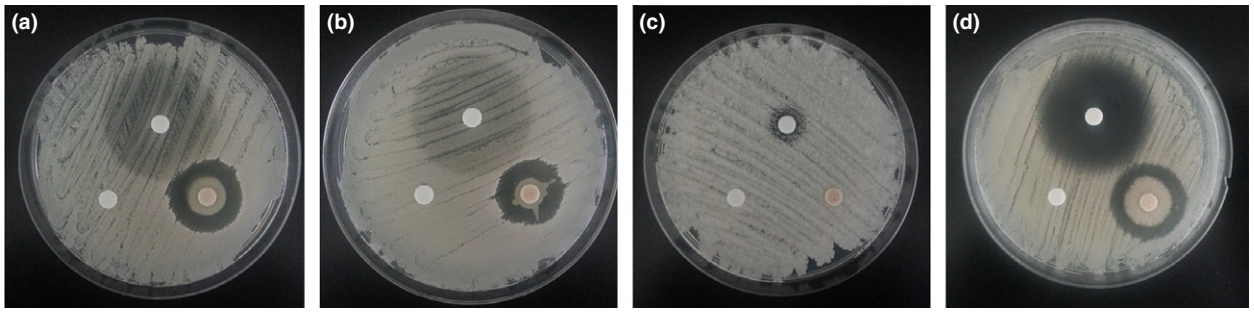

Figure 1 Disc diffusion test of *Bacillus subtilis* R0179 against *Candida* isolates. The disc diffusion test results are for *Candida albicans* ATCC 90028 (a), *C. albicans* SC5314 (b), *Candida krusei* ATCC 6258 (c) and *Candida parapsilosis* ATCC 22019 (d). For each agar plate, the upper disc was impregnated with fluconazole, the bottom-right with *B. subtilis* and the bottom-left disc was left blank.

the circular zones of *C. albicans* ATCC 90028 and five clinical *C. albicans* isolates are summarized in Table 1. The circular zone of growth inhibition induced by *B. subtilis* is much clearer than that induced by fluconazole, which indicating that *B. subtilis* may have direct fungicidal effect compared to fluconazole, which has a low fungicidal potential.

Cell viability determination by CLSM

CLSM revealed a remarkable reduction in *Candida* cells in the Flz-treated group ($P < 0{\cdot}001$) and the *B. subtilis*-treated group ($P < 0{\cdot}001$), compared to the control group (Figs 2 and 3a). We also quantified the percentage of dead yeast cells according to their distinct fluorescence emissions by visual counting (Figs 2 and 3b) (Jin *et al.* 2005). The percentage of dead *Candida* cells was significantly increased in the Flz group ($P < 0{\cdot}001$) and the *B. subtilis* ($P < 0{\cdot}05$) group compared to the blank group. *Bacillus subtilis* prevented the formation of a biofilm through the inhibition of hyphae formation, which led to a decrease in the yeast cell population.

Table 1 Inhibition induced by *Bacillus subtilis* of the growth of *Candida albicans* ATCC 90028 and five clinical *C. albicans* isolates obtained from the oral cavity (mm)

	Fluconazole		*B. subtilis*	
Candida species	Mean ± SD (mm)	95%CI	Mean ± SD (mm)	95%CI
C. albicans ATCC 90028	38·7 ± 0·6	37·2–40·1	27·7 ± 2·1	22·5–32·8
C.a 1	37·7 ± 1·5	33·9–41·5	26·7 ± 2·9	19·5–33·8
C.a 2	38·0 ± 2·0	33·0–43·0	24·0 ± 2·0	19·0–29·0
C.a 3	40·0 ± 1·0	37·5–42·5	26·7 ± 1·5	22·9–30·5
C.a 4	40·0 ± 1·0	37·5–42·5	29·0 ± 2·6	22·4–35·6
C.a 5	40·3 ± 1·5	36·5–44·1	26·3 ± 1·1	23·5–29·2

Morphological change of *Candida albicans* by SEM

Shrinkage and deformation of *C. albicans* cells was observed when co-cultured with *B. subtilis*. This was caused by membrane collapse. As is shown in the SEM images (Fig. 4), *Candida* cells in the blank control group were round and plump (Fig. 4a). However, in the Flz group (Fig. 4d) and the *B. subtilis* (Fig. 4b) group, many *Candida* cells appeared to have shrunk and were deformed.

Bacillus subtilis protects EHOM from damage caused by *Candida albicans*

Fungal invasion of this tissue was consistent with large-scale tissue damage and the epithelial layer was totally separated from the fibroblast layer. In the control group, abundant *C. albicans* (Fig. 5a) covered most of the surface of untreated EHOM. However, fewer *C. albicans* organisms were observed on *B. subtilis*-treated EHOM (Fig. 5b), which exhibited more minor tissue damage than was evident in the control group.

Iturin A can be detected in *Bacillus subtilis* R0179 supernatant by UPLC/MS

Peaks in the chromatograms were identified by comparing their migration times with Iturin A standard sample and Iturin A secreted by *B. subtilis* R0179. Figure 6 shows the peaks of Iturin A in standard and isolated from *B. subtilis* R0179 supernatant (Fig. 6). Iturin A can be detected in *B. subtilis* R0179 supernatant. The secretion of the antifungal agent Iturin A by *B. subtilis* R0179 maybe the main antifungal mechanism against *Candida* species.

Discussion

Candida species are the major cause of oral and oesophageal infections (Webb *et al.* 1998). Patients with AIDS,

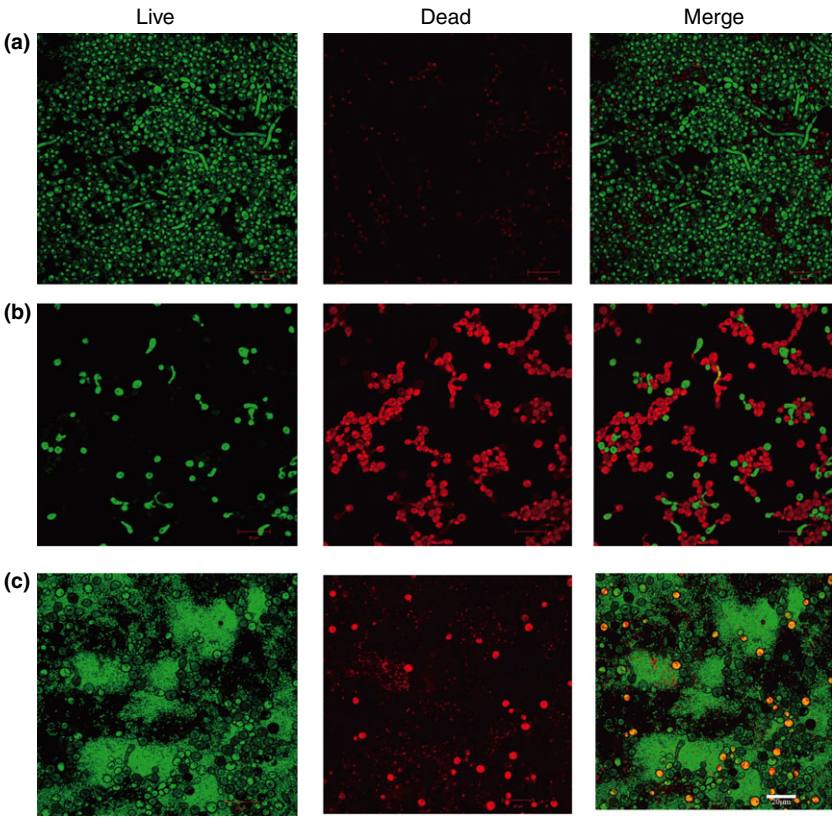

Figure 2 CLSM images of SYTO9 and PI stained *Candida albicans* SC5314 exhibiting differential Live/Dead staining patterns in response to the various treatments. (a) Blank control; (b) *C. albicans* with fluconazole (16 μg ml^{-1}); (c) *C. albicans* with *Bacillus subtilis* (10^9 CFU ml^{-1}). The extensive green dye in the c images is *B. subtilis* R0179. All scale bars represent 20 μm.

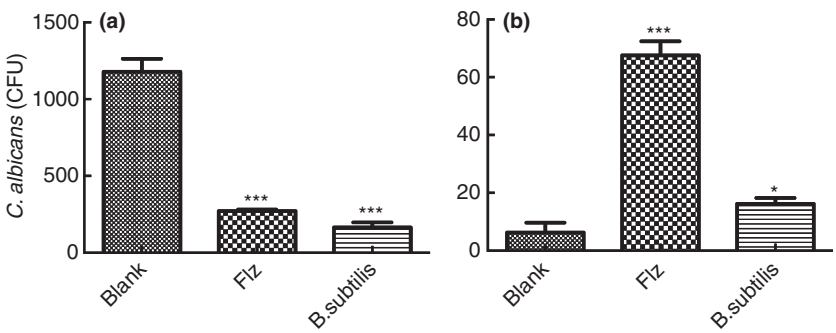

Figure 3 The total number of yeast cells and the percentage of cell death as determined by CLSM. (a) The total number of *Candida* cells (CFU) in response to the various treatments; (b) The percentage of dead *Candida* cells in response to the various treatments. *P* value < 0·05 (noted with *), *P* value < 0·001 (noted with ***) in comparison with blank control.

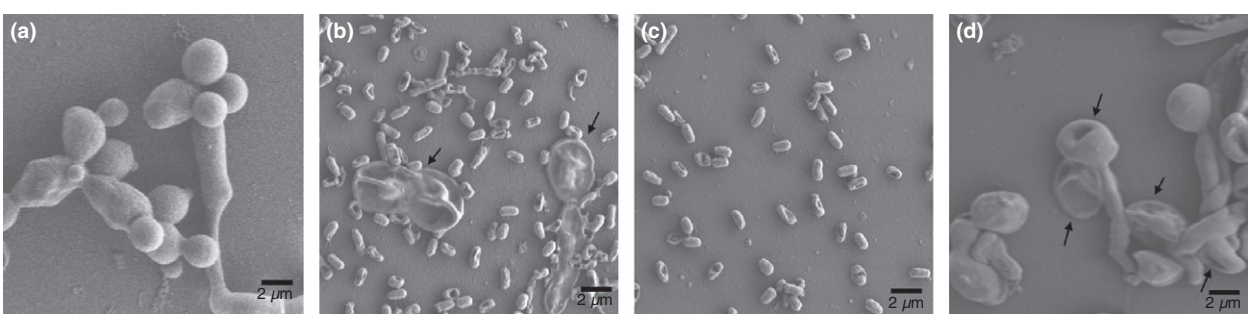

Figure 4 SEM images of *Candida albicans* cells following the various treatments. (a) *C. albicans* (10^6 CFU ml^{-1}); (b) *C. albicans* with *Bacillus subtilis* (10^9 CFU ml^{-1}); (c) *B. subtilis* (10^9 CFU ml^{-1}); (d) *C. albicans* with fluconazole (16 μg ml^{-1}). All scale bars represent 2 μm.

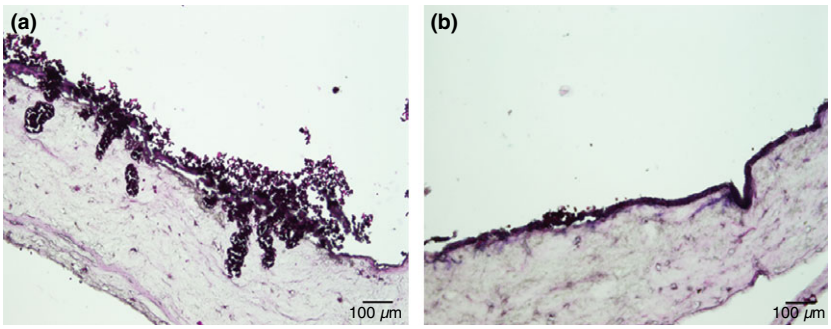

Figure 5 PAS-stained paraffin sections of three-dimensional models of the oral mucosa cocultured with *Candida albicans* ATCC 90028 for 24 h. (a) Blank control; (b) *C. albicans* with *Bacillus subtilis* (10^9 CFU ml^{-1}). All scale bars represent 100 μm.

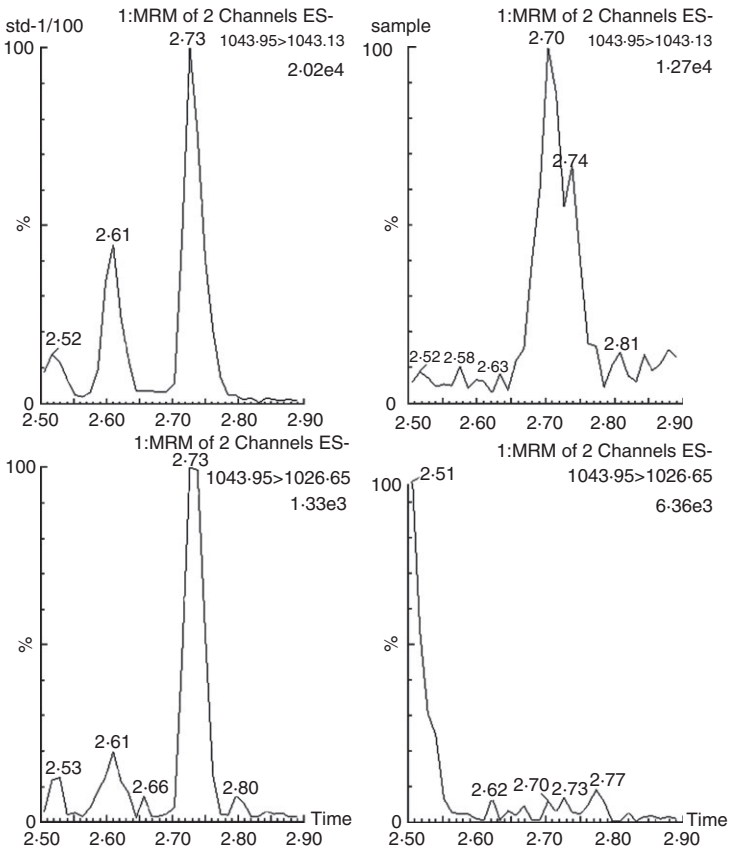

Figure 6 UPLC/TOF MS chromatograms for Iturin A standard sample (left panels) and Iturin A secreted by *Bacillus subtilis* R0179 (right panels).

hypo-salivation and diabetes mellitus, as well as those with poor oral hygiene have the greatest risk of developing oropharyngeal candidiasis (Ishijima *et al.* 2012). More importantly, *Candida* species have gained an increased resistance towards common antifungal drugs (Donlan and Costerton 2002). As a result, new strategies and agents are needed to prevent infections caused by these pathogens.

Some probiotic strains, such as *Lactobacilli*, have been used to treat and prevent vaginal fungal infections and it has been recommended that they be used in recurrent vulvovaginal candidiasis (RVCC) (Hilton *et al.* 1995). However, whether probiotics can be as effective in treating oral candidiasis remains a question. In this study, we aimed to detect the antifungal activity of probiotic products against oral *Candida* isolates *in vitro*. We found that *B. subtilis* might act as a potential candidate for the prevention and treatment of oral candidiasis.

Bacillus species have been used as probiotics for at least 50 years with the Italian product Enterogermina® registered in 1958 as an over-the-counter medication (Mazza 1994; Hong *et al.* 2005). Scientific interest in *Bacillus* species as probiotics, though, has only arisen in the last 15 years. *Bacillus* species, including *B. subtilis* are

important in the biological control of a number of plant and animal diseases (Chaurasia *et al.* 2005; Anderson *et al.* 2013). A few studies have indicated that *B. subtilis* might also be potentially applied to treat oral diseases. One study (Tsubura *et al.* 2009) demonstrated that a *B. subtilis* mouth wash had a positive effect on human patients with periodontitis and it was hypothesized that *B. subtilis* in mouth wash would adhere to and populate the periodontal tissue in conjunction with other oral bacteria and exhibit beneficial effects. Another study (Tsubura *et al.* 2012) demonstrated that a tablet (VITALREXTM (VL)) containing *B. subtilis* DB9011 is an effective oral probiotic material for patients with periodontitis. Our unpublished data show that *B. subtilis* can be isolated 24 h later after using Medilac-Vita mouthwash, which indicates that the adhesive property of *B. subtilis* to oral mucosa might be an important part of the probiotic functions. However, the mechanism by which *B. subtilis* adheres to oral epithelium needs to be further studied.

EHOM is well-organized and stratified tissue in which epithelial cells can express proliferating keratins such as Ki-67, K14 and K19, as well as differentiating keratin (K10). EHOM is also able to secrete IL-1β, IL-8, TNF-α, gelatinase-A and gelatinase-B (Rouabhia and Deslauriers 2002). In this study, an EHOM model has been used to study oral candidiasis, which highly resembles the native oral mucosa. It was revealed that *B. subtilis* R0179 was found to significantly inhibit the growth of *C. albicans* in co-culture and with EHOM. *Bacillus subtilis* treatment of EHOM tissue resulted in fewer *Candida* cells and less tissue damage compared to controls, which is consistent with the *in vitro* inhibitory effect. This study was the first time EHOM has been used to study the interaction between *C. albicans* and *B. subtilis*. The findings provided initial evidence of the *in vitro* anti-*Candida* activity of *B. subtilis*.

To date the exact mechanism by which *B. subtilis* inhibits *Candida* growth is not fully understood. Studies suggest that probiotics might prevent *Candida* growth through multiple mechanisms (Elahi *et al.* 2005). It was reported that *B. subtilis* has antifungal properties and some strains of *B. subtilis* can secrete antifungal agents including Iturin A, Surfactin and Fengycin (Tabbene *et al.* 2011). In our study, the secretion of Iturin A by *B. subtilis* R0179 was detected by UPLC/MS. Shrinkage and deformation of *Candida* cells were observed using SEM. This was caused by the leakage of intracellular plasma. These morphological changes may possibly have been caused by Iturin A, given its ability to pass through the cell wall and disrupt the plasma membrane (Thimon *et al.* 1995).

Candida species can be found in the oral cavity, intestinal tract, vagina and skin. *Candida* may affect other parts of the body. A study shows that infection of vulvovaginal candidiasis is highly associated with the concurrent infection of intestinal *Candida* (Lin *et al.* 2011). Control of oral *Candida* by probiotics might be beneficial for intestinal and vaginal infection of by *C. albicans*.

Bacillus subtilis R0179 survives passage through the human gastrointestinal tract by oral consumption and is well tolerated by healthy adults at intakes of from 0·1 to 10×10^9 CFU day^{-1}. *Bacillus* spores are able to withstand the antimicrobial properties of the gastrointestinal tract and begin to germinate and colonize the large intestine where they can potentially have beneficial effects (Hanifi *et al.* 2015). This provides evidence for the possibility that *B. subtilis* might colonize in the oral cavity, as the environment in the oral cavity is more moderate than that in intestinal tract. *Bacillus subtilis* might control *Candida* species in the oral cavity and the intestinal tract at the same time. More evidence is needed to confirm this hypothesis.

In summary, the current *in vitro* study demonstrates that *B. subtilis* R0179, isolated from a probiotic product treating gastrointestinal disorder, exhibited anti-*Candida* activity, and therefore could be a promising alternative probiotic treatment for oral candidiasis. Further studies are needed to confirm these findings.

Acknowledgements

The study was funded by grants of the National Natural Science Foundation of China [grant number 81000441, 81570985].

Conflict of Interest

The authors declare that they have no conflict of interest.

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