**Lactobacillus fermentum** Ess-1 with unique growth inhibition of vulvo-vaginal candidiasis pathogens

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The aim of this study was to characterize human isolates of **Lactobacillus** species for their capacity to interfere with the growth of different strains of **Candida** species *in vitro* in the search for a potential probiotic. Growth inhibition of **Candida** species was screened using an agar-overlay method. Inhibiting strains were selected to assay the effect of a cell-free **Lactobacillus** culture filtrate (LCF) on the growth of isolates of **Candida albicans** and **Candida glabrata**. A total of 126 human **Lactobacillus** isolates was investigated. Eighteen isolates significantly inhibited the growth of **C. albicans** on agar. The LCF of one of these strains showed strong inhibition of both **C. albicans** and **C. glabrata**. This strain was genetically identified as **Lactobacillus fermentum** and designated L. fermentum Ess-1. Further tests to evaluate the probiotic potential of this strain indicated that L. fermentum Ess-1 strain is a promising probiotic for use in clinical trials to treat and prevent vulvo-vaginal candidiasis.

**INTRODUCTION**

Vulvo-vaginal candidiasis (VVC) is characterized by overgrowth of yeast species in the vulva and vagina. **Candida albicans** accounts for 90% of all VVC infections, and other yeast species causing VVC are often categorized as non-**candida** species (Paulitsch et al., 2006). An increased prevalence of non-**candida** species, foremost **Candida glabrata**, has been detected in recent years and has led to increased medical concern (Martens et al., 2004; Spinillo et al., 1997). VVC caused by **C. glabrata** is more difficult to diagnose by microscopy because of the lack of hyphae, and needs to be verified by culture. There is also no clear strategy regarding the treatment of VVC caused by this species. **C. glabrata** has low susceptibility to azoles, increasing the need for new treatment strategies (Sobel et al., 2003).

Lactobacilli are natural inhabitants of the vulvo-vaginal microflora and are believed to have a central role in the suppression of potential pathogens. Lactobacilli administered to the genital tract have a prominent role as a prophylactic aimed at improving the genital microfloral defence against bacterial infections (Juarez Tomas et al., 2003; Kaewsrichan et al., 2006; Voravuthikunchai et al., 2006). Strains that exert a health benefit to the host are classified as probiotics. Probiotic strains that can be used for the treatment of VVC should be able to produce metabolites that are fungistatic for **C. albicans** and **C. glabrata**. Results where metabolites from **Lactobacillus** isolates strongly affect **Candida** growth *in vitro* have only been published in a few reports and relate to **C. albicans** in particular (Oikkers et al., 1999; Strus et al., 2005b; Wynne et al., 2004). To the best of our knowledge, no studies have demonstrated an effect of **Lactobacillus** isolates on the growth of **C. glabrata**. The aim of the present study was to find a probiotic candidate that inhibited the growth of both **C. albicans** and **C. glabrata**.

**METHODS**

**Bacteria and yeast isolates.** A total of 126 human **Lactobacillus** isolates was isolated from the forehead, throat and teeth of healthy adult volunteers and from faecal samples from newborn infants obtained 3–5 days after birth. Swab samples obtained from the donors were cultured on a **Lactobacillus**-selective agar [de Man, Rogosa and Sharpe (MRS) agar; Merck] at 37 °C for 48 h with 5% CO2.

All isolates were screened for yeast growth inhibiting capacity. The four target **Candida** isolates were vaginal isolates from women with VVC (**C. albicans** 702, **C. albicans** A, **C. glabrata** 1 and **C. glabrata** 2). In addition, two reference strains (**C. albicans** CCUG 44135 and **C. glabrata** CCUG 44136), originally isolated from the vagina of healthy females, were used. In screening procedures, the previously characterized strain **Lactobacillus plantarum** LB931 (Rönnqvist et al., 2005) was used as a reference, together with additional **Lactobacillus** strains with various fungistatic properties. These reference strains were **Lactobacillus casei** Shirotu (Yakult), **L. casei** Defens (Danone), **Lactobacillus fermentum** ITM6E, **Lactobacillus brevis** ITM1F (Microbiolgy Institute of the Catholic University of Picenza).
growth of Candida and hence was used as a negative control in the experiment assessing the effect of pH on inhibition described below.

**Initial screening using a modified agar overlay technique.** Each Lactobacillus isolate was cultured in MRS broth (Merck) for 20 h at 37 °C. The broth was then added to wells in a Bertani tray and stamped onto an MRS agar plate using a sterile Steer’s steel pin replicator. The agar plate was incubated under anaerobic conditions at 37 °C for 24 h. Thereafter, 12 ml Sabouraud dextrose agar (LAB M) was poured onto the MRS agar and allowed to solidify. Approximately 6 log_{10} C. albicans organisms were seeded onto the Sabouraud dextrose agar and the plate was incubated aerobically at 37 °C for 24 h. Candida growth inhibition on the spot located above each Lactobacillus isolate was evaluated, and Lactobacillus isolates inhibiting growth equal to or greater than L. plantarum LB931 were selected for further secondary screening.

**Secondary screening by cell-free filtrate inhibition.** Each Lactobacillus isolate was cultured in 4 ml dMRSS broth (MRS broth without the addition of sodium acetate; Stiles et al., 2002) for 20 h at 37 °C and then centrifuged at 1900 g for 10 min at 10 °C. The supernatant was sterilized by passing through a 0.22 μm pore-size filter and the pH was monitored using a pH meter (Metrohm). This filtrate was designated Lactobacillus cell-free filtrate (LCF). Aliquots (750 μl) of LCF were transferred to the wells of a 48-well microtitre plate (Sarstedt), air-dried at 45 °C for 20 ± 2 h and resuspended in 250 μl sterile distilled water. A total of 200 μl of each concentrated LCF was transferred to a well of a 96-well microtitre plate. Controls contained fresh dMRSS or dMRSS broth with the pH adjusted to the same pH as the lowest monitored LCF pH, and otherwise treated as the LCF samples. Three isolates each of C. albicans and C. glabrata were inoculated in the wells at a final concentration of 4.5 log_{10} c.f.u. ml^{-1} and grown at 37 °C. After 24 h, the growth inhibition score was determined according to the scale shown in Fig. 1. Inhibition was scored from 0 to 10, where LCFs with lack of inhibition equal to the controls were assigned a score of 0, and LCFs showing complete inhibition of Candida growth were assigned a score of 10. All samples were analysed in duplicate and the inhibition score was determined by two independent investigators who were blind to the origin of the sample. The inhibition assay was repeated twice and the results of each isolate were presented as the mean of the determined values.

**API typing and genetic typing of Lactobacillus isolates.** Identification to the species level was carried out using the API 50 CHL system (bioMérieux), following the manufacturer’s instructions. Data from the fermentation tests were analysed using API LAB PLUS software. Genetic typing was carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Gent, Belgium) by partial sequence analysis of the 16S rRNA gene.

**Effect of pH on inhibition with LCF.** The pH of two LCFs was adjusted to 5.0 and 7.0 using 2 M sodium hydroxide. A third test tube containing LCF at the original pH of 3.7 was not adjusted. The LCFs were sterilized at +8 °C for 1 h and centrifuged at 2400 g at 10 °C for 15 min. The supernatant was sterilized through a 0.22 μm pore-size sterile filter and the filtrate was transferred to 96-well microtitre plates. C. albicans CCUG 44135 was added to a final concentration of 4.5 log_{10} c.f.u. ml^{-1}. The plates were incubated at 37 °C for 24 h and visually evaluated by two investigators as described in the secondary screening process. All samples were analysed in duplicate.

**Survival following freeze-drying and storage at 37 °C.** L. fermentum Ess-1 was cultured in MRS broth for 18 h at 37 °C, centrifuged at 3100 g at 6 °C for 22 min and the pellet concentrated tenfold in 20% trehalose (Bröste). The suspension was frozen at −55 °C and thereafter freeze-dried in a Hetoiss freeze-drier (Heto Birkerod) for 48 h. The end product was pulverized with a mortar and stored in a sealed plastic tube at 37 °C for 11 weeks. The number of viable bacteria was determined at weeks 0, 2, 5 and 11.

**Vaginal insertion of L. fermentum Ess-1.** Lyophilized powder containing L. fermentum Ess-1 was placed into gelatin capsules (size 2; Apoteket) at a concentration of ~10^9 c.f.u. per capsule. Four healthy, pre-menopausal volunteers (aged 19–28 years) introduced one capsule to the vagina 1 week prior to menses. Vaginal samples were taken prior to vaginal insert and the day after the last bleeding day by turning a sterile cotton swab three times 3 cm above the introitus. The cotton swab was stored in MRS broth at 8 °C for not more than 48 h until quantification of L. fermentum Ess-1. The sample was cultured on Rogosa agar plates with 128 mg vancomycin l^{-1} to minimize the growth of endogenous lactobacilli. Colonies were counted and the number of c.f.u. ml^{-1} was determined. The study was approved by the Regional Ethical Review Board in Umeå, Sweden.

**Susceptibility to antifungal drugs.** The susceptibility of L. fermentum Ess-1 to antifungal substances was tested using E-tests (AB Biodisk) with amphotericin, caspofungin, fluconazole, flucytocine, itraconazole, ketoconazole, posaconazole and voriconazole. A total of 100 μl L. fermentum Ess-1 and 0.9 % sodium chloride suspension corresponding to 1 McFarland standard was seeded onto agar plates containing Iso-Sensitest agar (Oxoid) with the addition of 5% horse blood. After 15 min, the E-tests were applied to the agar and the plates were incubated for 24 h with 5% CO₂ at 37 °C. The susceptibility results were determined according to the manufacturer’s instructions.

**Growth inhibition effect on vaginal lactic acid bacteria (LAB) isolates.** A previously described method (Ronqvist et al., 2005) was carried out, with some modifications, to evaluate whether metabolites produced by L. fermentum Ess-1 inhibited the growth of other LAB. Briefly, L. fermentum Ess-1 was inoculated into a 25 ml modified MRS agar layer (sodium acetate concentration adjusted to 0.29 M) to a final concentration of 7.5 log_{10} c.f.u. ml^{-1}. The plate was incubated for 24 h and an additional layer of MRS agar with the addition of KH₂PO₄ (final concentration 0.17 M) was poured on top of the first MRS agar layer. Ten LAB isolates [L. plantarum (4), L. rhamnosus (1), Lactobacillus paraseii (1), Lactobacillus celsiensis (1), Pediococcus pentosaceus (2) and Pediococcus acidilactici (1)] were stumped onto the solidified and dried agar using a Steer’s steel-pin replicator. The plate was incubated at 37 °C in 5% CO₂ for 24 h. The results were assessed as ‘inhibition’ (no or weak visual growth) or ‘no inhibition’ (strong growth).

**RESULTS AND DISCUSSION**

Use of probiotic bacteria for the treatment of disorders of the urogenital sphere has focused mainly on urinary tract
infections and bacterial vaginosis. The possibility of preventing VVC using probiotics has been less extensively investigated (Reid & Bruce, 2003). In bacterial vaginosis, there is always a quantitative reduction in the number of endogenous vaginal lactobacilli. Trials aiming to restore dominance of the Lactobacillus flora by oral and/or local application of probiotic strains have not been conclusive, indicating that not only quantitative but also qualitative properties of the normal flora are essential (Wilson, 2004). In VVC, there is no such clear relationship between Candida overgrowth and paucity of lactobacilli in the vaginal flora (Demirezen, 2002). Given the clinical impact of VVC, alternative treatment strategies for this condition should be explored. Qualitative properties, such as inhibition of yeast growth, could be an important feature of a candidate probiotic strain for the treatment of VVC.

To determine a probiotic strategy for the treatment of VVC, we focused our search on Lactobacillus to determine a probiotic strategy for the treatment of of a candidate probiotic strain for the treatment of VVC. In VVC, there is no such clear relationship between Candida overgrowth and paucity of lactobacilli in the vaginal flora (Demirezen, 2002). Given the clinical impact of VVC, alternative treatment strategies for this condition should be explored. Qualitative properties, such as inhibition of yeast growth, could be an important feature of a candidate probiotic strain for the treatment of VVC.

**Screening and typing**

Previous work from our own laboratory has shown a total lack of C. albicans growth inhibition by vaginal Lactobacillus isolates (Rönnqvist et al., 2005). Those findings taken together with the findings of Strus et al. (2005a) were used to design the present study encompassing the screening of Lactobacillus strains isolated from a number of different sites in order to maximize variation in the properties among strains. The 126 human Lactobacillus strains used were isolated from teeth (n=82), throat (n=5), forehead (n=3) and neonatal faeces (n=36). In the initial screening process, 18/126 Lactobacillus isolates (14.3%), the majority of which were collected from the oral tract of healthy adults, showed a detectable inhibitory effect on C. albicans growth comparable to or exceeding that of L. plantarum LB931. In addition, C. albicans inhibition was found in the two reference strains L. rhamnosus IMPC19 and L. rhamnosus GG. These 20 Lactobacillus isolates, together with L. plantarum LB931, were selected for secondary screening. Fig. 2 illustrates the growth inhibition scores for each Lactobacillus isolate against three different C. albicans isolates and three different C. glabrata isolates. Of the 21 Lactobacillus isolates tested in the secondary screening, only 1 proved to have a strong inhibitory activity against both Candida species. This strain was originally isolated from the throat of a healthy person and was typed as L. fermentum. It was designated L. fermentum Ess-1 and is the first Lactobacillus strain described with significant growth inhibition activity against both C. albicans and C. glabrata.

**Initial characterization of L. fermentum Ess-1**

Studies were carried out to evaluate the potential probiotic use of L. fermentum Ess-1 in vaginal health. As such, the stability of the metabolites inhibiting Candida growth at different vaginal pH levels was of interest. The vaginal pH among healthy women, as well as in women with VVC, is normally around 4.5 (Sobel & Chaim, 1996), but varies among individuals. No sign of decreased activity in pH-adjusted filtrate between pH 3.7 and 7.0 was found
(Table 1), indicating that the active metabolites were stable within the clinically relevant pH range that occurs in the vagina and on vulvar skin (Runeman et al., 2004).

Furthermore, probiotic lactobacilli should not interfere with the normal vulvo-vaginal LAB flora. Nine out of ten tested LAB were not inhibited by L. fermentum Ess-1 (not shown). Only one strain of L. cebalbiosa, a rather rare isolate of the vaginal flora (Vasquez et al., 2002), was affected by metabolites produced by L. fermentum Ess-1.

Kilic et al. (2005) studied the susceptibility of lactobacilli against two antifungal drugs: isoconazole and oxiconazole. It was concluded that lactobacilli harbour a natural resistance against these drugs. Our findings affirmed this conclusion. L. fermentum Ess-1 was resistant to all antifungal drugs tested, except for flucytosine (0.125 μg ml⁻¹). C. glabrata harbours a relatively low susceptibility to azoles in vitro but shows high susceptibility to flucytosine (Richter et al., 2005). The first report of intravaginal flucytosine treatment for women positive for C. glabrata showed a 90% cure rate (Sobel et al., 2003). However, long-term use of flucytosine to prevent re-infection or relapse was not recommended. At this step, an intravaginal supply of L. fermentum in infection or relapse was not recommended. At this step, an

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Inhibition scores for LCF against C. albicans CCUG 44135 at three different pH levels

<table>
<thead>
<tr>
<th>pH</th>
<th>Ess-1</th>
<th>LB99</th>
<th>LB931</th>
<th>dMRSs</th>
</tr>
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<tbody>
<tr>
<td>3.7</td>
<td>8.5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.0</td>
<td>7.5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>8</td>
<td>2</td>
<td>0.5</td>
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Growth inhibition was scored from 0 (no visual inhibition) to 10 (complete visual inhibition), as shown in Fig. 1. so was ± 0–0.41.

Conclusions

Metabolites produced by L. fermentum Ess-1 show exceptional fungistatic properties against the two most common yeast species associated with VVC, C. albicans and C. glabrata. L. fermentum Ess-1 has great potential to be used as a probiotic to treat symptomatic VVC or to prevent recurrent VVC infection.

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REFERENCES


