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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-*albicans* species (Paulitsch *et al.*, 2006). An increased prevalence of non-*albicans* 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 habitants 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 (Okkers *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*.

Abbreviations: LAB, lactic acid bacteria; LCF, *Lactobacillus* cell-free filtrate; VVC, vulvo-vaginal candidiasis.

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 % CO_2.

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* Shirota (Yakult), *L. casei* Defensis (Danone), *Lactobacillus fermentum* ITM6E, *Lactobacillus brevis* ITM1F (Microbiology Institute of the Catholic University of Piacenza,

Italy), *Lactobacillus rhamnosus* IMPC19 (both from the Institute of Sciences of Food Production, CNR, Bari, Italy), *L. rhamnosus* GG (ATCC 53103) and *L. plantarum* 299v (DSM 9843). *L. fermentum* LB99 is an isolate that does not produce metabolites that inhibit the 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.

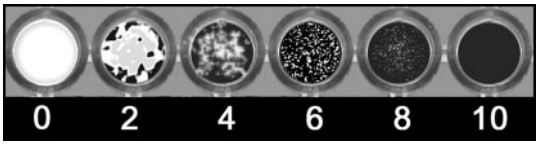

Fig. 1. Determination of *Candida* growth inhibition scores. No visual inhibition was scored as 0 and complete visual inhibition as 10.

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 stabilized at +8 °C for 1 h and centrifuged at 2480 ***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 Hetosicc freeze-drier (Heto Birkerød) 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 $\sim 10^9$ 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 % CO_2 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 (Rönnqvist *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 KH_2PO_4 (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 paracasei* (1), *Lactobacillus cellobiosus* (1), *Pediococcus pentosaceus* (2) *and Pediococcus acidilactici* (1)] were stamped onto the solidified and dried agar using a Steer's steel-pin replicator. The plate was incubated at 37 °C in 5 % CO_2 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* strains that produced metabolites that inhibited the growth of *Candida* species. We suggest that promising probiotic bacteria should be evaluated with respect to growth inhibition of both *C. albicans* and *C. glabrata*, as these are the most frequently isolated VVC pathogens. To the best of our knowledge, only one *in vitro* study regarding the growth inhibitory effect of lactobacilli on these *Candida* species has been published (Strus *et al.*, 2005a), where 25 vaginal *Lactobacillus* isolates of different species were studied. Isolates typed as *Lactobacillus delbrueckii* exhibited the strongest inhibitory activity against *C. albicans*, but none of the isolates showed growth inhibition activity against *C. glabrata*.

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

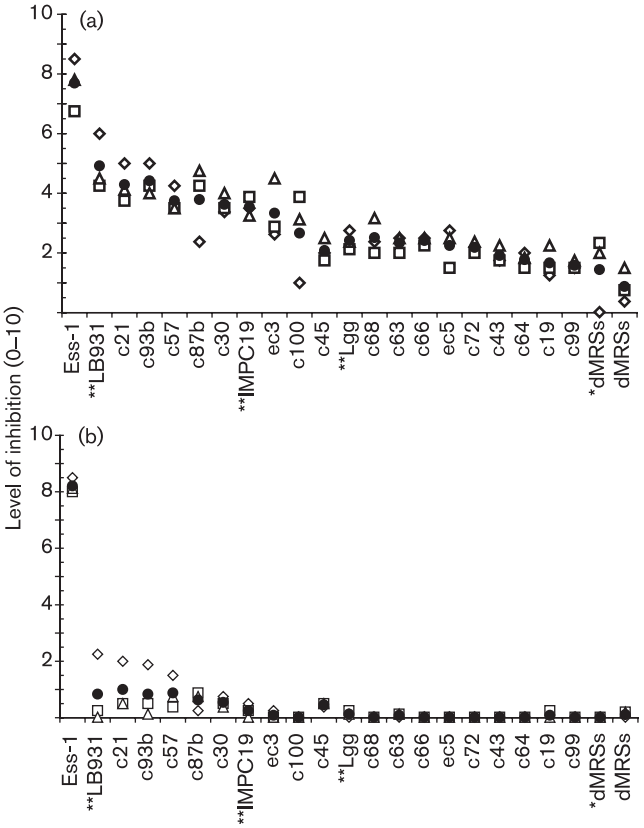

Fig. 2. Secondary screening inhibition scores for LCF against *C. albicans* (a) and *C. glabrata* (b). *C. albicans* or *C. glabrata* isolates were inoculated into LCFs from different *Lactobacillus* isolates. After 24 h, the growth inhibition score for each *Lactobacillus* isolate was determined according to Fig. 1 on a scale of 0–10. The break point of each *Candida* isolate is shown as follows: (a) ◇, *C. albicans* CCUG 44135; □, *C. albicans* 702; △, *C. albicans* A; and (b) ◇, *C. glabrata* CCUG 44136; □, *C. glabrata* 1; △, *C. glabrata* 2. ●, The mean of all three isolates; *, dMRSs broth with pH adjusted to the lowest monitored pH of all filtrates (pH 3.8); **, reference strains.

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. cellobiosus*, 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 flucytocine treatment for women positive for *C. glabrata* showed a 90 % cure rate (Sobel *et al.*, 2003). However, long-term use of flucytocine to prevent re-infection or relapse was not recommended. At this step, an intravaginal supply of *L. fermentum* Ess-1 could be a promising intervention to prevent *C. glabrata* overgrowth in the vulva and vagina. For this purpose, it is of great importance that *L. fermentum* Ess-1 survives and stays viable when supplied to the vagina. A pilot study was carried out with vaginal insertion of *L. fermentum* Ess-1. Four volunteers inserted capsules containing a lyophilized powder of *L. fermentum* Ess-1 the week prior to menses. *L. fermentum* Ess-1 was detected in all of the women after menses and the mean number of identified *L. fermentum* Ess-1 was 3.7 $\log_{10}$ c.f.u. per sample. The samples with the highest and lowest numbers contained 6.2 and 1.3 $\log_{10}$ c.f.u. per sample, respectively. These results strengthen the potential use of this strain as a vaginal probiotic. Further tests showed that the bacterial number in the lyophilized powder was reduced from 10.0 to 8.9 $\log_{10}$ c.f.u. g^{-1} after 11 weeks of storage in sealed tubes at 37 °C. Survival at room temperature is believed to be considerably longer, which would facilitate the distribution and storage of sealed products containing *L. fermentum* Ess-1. Future studies to evaluate the possible clinical benefits of *L. fermentum* Ess-1 in women with VVC are essential and have been initiated.

Table 1. Inhibition scores for LCF against *C. albicans* CCUG 44135 at three different pH levels

Growth inhibition was scored from 0 (no visual inhibition) to 10 (complete visual inhibition), as shown in Fig. 1. SD was ±0–0.41.

pH	Ess-1	LB99	LB931	dMRSs
3.7	8.5	2	0	0
5.0	7.5	2	0	0
7.0	8	2	0.5	0

Conclusion

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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