Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Improved treatment of vulvovaginal candidiasis with fluconazole plus probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14

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Keywords
Candida sp., fluconazole, *L. reuteri* RC-14, *L. rhamnosus* GR-1, probiotic, vulvovaginal candidiasis.

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2008/0857: received 19 May 2008, revised 26 July 2008 and accepted 28 July 2008

doi:10.1111/j.1472-765X.2008.02477.x

Abstract

Aims: To determine the ability of probiotic lactobacilli to improve the treatment of vulvovaginal candidiasis (VVC) using a randomized, double-blind and placebo-controlled trial.

Methods and Results: Fifty-five women diagnosed with VVC by vaginal discharge positive for *Candida* spp. (according to culture method) associated with at least one of the symptoms (itching and burning vaginal feeling, dyspareunia and dysuria), were treated with single dose of fluconazole (150 mg) supplemented every morning for the following 4 weeks with two placebo or two probiotic capsules (containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14). At 4 weeks, the probiotic treated group showed significantly less vaginal discharge associated with any of the above mentioned symptoms (10·3% *vs* 34·6%; $P = 0{\cdot}03$) and lower presence of yeast detected by culture (10·3% *vs* 38·5%; $P = 0{\cdot}014$).

Conclusion: This study has shown that probiotic lactobacilli can increase the effectiveness of an anti-fungal pharmaceutical agent in curing disease.

Significance and Impact of the Study: This novel finding of probiotic lactobacilli augmenting the cure rate of yeast vaginitis, not only offers an alternative approach to a highly prevalent condition that adversely affects the quality of life of women around the world, but also raises the question of how this combination works.

Introduction

Vulvovaginal candidiasis (VVC) afflicts an estimated 75% of sexually active women at least once in their life (Ferrer 2000) and of these, approx. 50% will develop a second episode, with 5% suffering recurrent VVC, defined as four or more episodes within 1-year period (Eckert *et al.* 1998; Paulitsch *et al.* 2006). VVC is the second most common cause of vaginal infections after bacterial vaginosis (BV) (Sobel 1985) and it adversely affects physical and emotional health, sexual and conjugal relationships (Chapple *et al.* 2000). In USA, diagnosis and treatment of VVC, together with lost productivity, resulted in an estimated cost of US$1·8 billion in 1995, which could rise to up to US$3·1 billion in 2014 (Foxman *et al.* 2000).

VVC is diagnosed by the presence of a thick, white vaginal discharge in association with vulvar itching, burning and/or dysuria (Sheary and Dayan 2005), in addition to normal vaginal pH (<4·5), presence of hyphae, pseudohyphae or budding yeasts visualized under optical

microscopy (×400 magnification) in wet mount preparations with 10% potassium hydroxide (KOH), visualization of fungi constituents after Gram-staining the vaginal sample and growth of the micro-organism in selective culture media (Eckert *et al.* 1998; Williams *et al.* 2001).

The treatment of VVC is reasonably effective with a range of oral and intravaginal agents, although patients often suffer from side effects, such as altered palate, nausea, diarrhea and vaginal burning. Two problems still exist: the subsection of women suffering recurrent VVC, and the fact that anti-fungal therapy is not designed to restore the normal microbiota of the vagina. Indeed, BV, a condition with an overall high prevalence (Allsworth and Peipert 2007) and associated with numerous complications (Morris *et al.* 2001) can result from the use of anti-fungal agents (Pawlaczyk *et al.* 2006). Vaginitis induced by non-*albicans* species is clinically indistinguishable from that caused by *Candida albicans*; in addition, such species are often more resistant to treatment (Bauters *et al.* 2002).

Based upon the theory that VVC is a result of, or causes, a disruption of the vaginal microbiota, attempts to prevent infection using lactobacilli have been reported (Williams *et al.* 2001). Pirotta *et al.* (2004) evaluated the role of orally or vaginally administered *Lactobacillus* to prevent vulvovaginitis after antibiotic treatment in a group of 278 nonpregnant women from Melbourne (Australia) and observed that the approach was not effective. However, they used a relatively unproven probiotic, and there is little evidence that lactobacilli *per se* can cure VVC.

In a previous study, we have shown that orally administered *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 could augment the cure of BV (Anukam *et al.* 2006). In the present study, the objective was therefore to assess whether probiotic lactobacilli improved the efficacy of fluconazole in patients with VVC.

Subjects, materials and methods

Probiotic strains

The strains *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were provided by Chr. Hansen, Horsholm, Denmark, in gelatin capsules manufactured under good manufacturing practices. Each capsule contained 1×10^9 viable cells of both strains. Gelatin capsules containing cellulose and magnesium stearate were used as placebo.

Ethics, subjects' recruitment and medical team

Each subject voluntarily signed an informed consent and answered a questionnaire using a format approved by the Ethics Review Board of the Centro de Saúde Escola da Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo (CSE-FMRP-USP) – Protocol no. 0146. This study was registered online at 'Comissão Nacional de Ética em Pesquisa' (CONEP) (document no. 070202), Brazil.

The inclusion criteria for entry into the study was subjects suffering from vaginal discharge associated with any of the following symptoms: itching and burning vaginal feeling, dyspareunia and dysuria, whose vaginal samples were positive for *Candida* spp. by culture method. The exclusion criteria included: pregnancy, HIV positive patients and those ones who were also positive for BV or trichomoniasis; use of systemic or intravaginal antibiotic or anti-fungal agents currently or within the past 2 weeks of the appointment, menses during samples collection and allergic responses to fluconazole.

Subjects were recruited and examined by the gynaecological team from the CSE-FMRP-USP and three other affiliated sites: Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto (HCFMRP)-USP, Sistema Integrado de Saúde (SIS)-USP and Centro de Saúde Vila Lobato (CSVL)-FMRP-USP, Brazil.

Clinical and laboratory procedures done during the gynaecology appointment

During the gynaecological consultation, the physician detected the presence of vaginal discharge and determined the presence of any of the following symptoms and signs (itching and burning vaginal feeling, dyspaureunia and dysuria). Vaginal pH was measured by contact of a pH indicator strip (Acilit pH 0–6, Merck, Darmstadt, Germany) in the lateral vagina wall, and three vaginal samples were collected with the use of a swab and an Ayres spatula. The samples were concomitantly evaluated by Gram-staining and wet mount preparation, to exclude the diagnosis of BV [characterized by the presence of 'clue cells' in Gram-staining of vaginal smears and the development of fishy odour (positive 'Whiff' test) respectively] (Amsel *et al.* 1983; Nugent *et al.* 1991) and to visualize fungal elements. The collected swab was used for seeding, isolation and identification of *Candida* micro-organisms.

Randomization of treatment and follow-up appointment

Subjects diagnosed with VVC were randomized to treatment with a single dose of fluconazole (150 mg) plus either two oral capsules of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or placebo once daily (every morning) for 28 days starting on the day of fluconazole use. Subjects

stated good adherence to the protocol and many patients returned the empty vials.

During the second visit, the presence of vaginal discharge was evaluated along with the presence of any symptoms and signs (itching and burning vaginal feeling, dyspaureunia and dysuria) and side-effects related to the drug and/or probiotic. Findings were blinded to all the investigators until the analyses had been completed.

Seeding, isolation and identification of *Candida* species

The collected swabs were immediately introduced into tubes containing a 0·85% (w/v) sodium chloride solution and the suspension was seeded in a selective and differential culture medium: CHROMagar® Candida (Probac, São Paulo, Brazil). The isolates were identified by biochemical tests (germinative tube and microculture, assimilation of sources of carbohydrates and nitrogen and also carbohydrate fermentation tests), according to Kurtzman and Fell (1998).

Statistical analysis

The chi-squared and Fisher's exact two-tailed tests were used to evaluate if there were differences between the various outcomes obtained from groups treated with anti-fungal medication supplemented with probiotic or placebo. Significance was set at a level of 5%. SAS software, ver. 9.1; SAS Institute Inc., Cary, NC, USA, was used for the statistical tests.

Results

A total of 68 patients were recruited at four different public health centres in the city of Ribeirão Preto, São Paulo State (Brazil) between September 2006 and April 2007. The women were diagnosed with VVC by the presence of vaginal discharge in association with at least one of the typical symptoms and signs of the disease (itching and burning vaginal feeling, dyspareunia and dysuria) and by the presence of *Candida* at least by one of the following methods: wet mount preparation, Gram-staining or culture. Then, only patients who had positive cultures for *Candida* were taken into consideration (55 subjects), as culture is believed to be the gold standard for diagnosis of VVC (Chatwani *et al.* 2007).

In one group, 29 subjects were randomized for treatment with single dose fluconazole and daily supplementation with probiotic capsules and, in a second group, 26 subjects were treated with fluconazole and placebo capsules. Compliance was excellent. The results of demographic and behavioural characteristics, as well symptoms, signs and microbiology findings are summarized in Table 1. *C. albicans* represented 93·3% of all isolates in the probiotic group, and 84·6% in the placebo group.

At day 28, more subjects treated with fluconazole and probiotics exhibited cure of VVC as determined by having no vaginal discharge, itching and/or burning vaginal feeling, dyspareunia and/or dysuria, and *Candida* recovered by culture method, when compared with subjects

Table 1 Demographic and behavioural characteristics, as well as symptoms, signals, and results of laboratory determinations from patients diagnosed with VVC: 29 women randomized to be treated with single dose of fluconazole (150 mg) and oral probiotic capsules daily in the morning during 4 weeks, and 26 women treated with fluconazole and placebo capsules

Observation	Probiotic group (n = 29)	Placebo group (n = 26)
Mean age (range)	29·1 ± 7·5 (16–46)	26·9 ± 7·8 (16–42)
Use of contraceptive methods	17 (58·6)	18 (69·2)
Use of hormonal contraceptive methods	12 (41·4)	14 (53·8)
Regular menses	19 (65·5)	20 (76·9)
Recurrent VVC (self-reported)	11 (37·93)	5 (19·2)
Vaginal discharge associated with at least one of the symptoms (itching and burning vaginal feeling, dyspareunia and dysuria)	29 (100·0)	26 (100·0)
Positive 'Whiff' test	1 (3·4)	1 (3·8)
Nugent BV score	0 (0·0)	0 (0·0)
Vaginal pH inferior to 4·5	27 (93·1)	25 (96·2)
Presence of *Candida* by culture method	29 (100·0)*	26 (100·0)†
Presence of *Candida* on wet mount preparations	28 (96·6)	23 (88·5)
Presence of *Candida* on vaginal Gram-staining smears	25 (86·2)	21 (80·8)

Results are expressed as absolute numbers and percentages respectively.

**C.* non-*albicans* was detected in 6·7% of samples: *C. glabrata* (one isolate) and *C. krusei* (one isolate).

†*C.* non-*albicans* was detected in 15·4% of samples: *C. glabrata* (one isolate), *C. parapsilosis* (two isolates) and *C. tropicalis* (one isolate).

Table 2 Findings after 4 weeks of treatment, comparing patients with VVC treated with single dose of fluconazole (150 mg) and supplemented with oral probiotic or placebo capsules daily for 4 weeks

Outcome	Fluconazole plus lactobacilli (n = 29)	Fluconazole plus placebo (n = 26)	P-vlaue
Vaginal discharge associated with at least one of the symptoms (itching and burning vaginal feeling, dyspareunia and dysuria)	3 (10·3)	9 (34·6)	0·030
Presence of *Candida* by culture method	3 (10·3)	10 (38·5)	0·014
Positive 'Whiff' test	1 (3·4)	2 (7·7)	0·598φ
Vaginal pH >4·5	2 (6·9)	1 (3·8)	>0·999φ
Nugent BV score	0 (0·0)	2 (7·7)	0·219φ

Results are expressed as absolute numbers and percentages respectively*.
*For proportions a chi-square test was used except where noted by φ. For these, Fisher's exact two-tailed test was used.

Table 3 Findings after 4 weeks of treatment, comparing patients with recurrent VVC (self-reported) treated with single dose of fluconazole (150 mg) and supplemented with oral probiotic or placebo capsules daily for 4 weeks

Outcome	Fluconazole plus lactobacilli (n = 11)	Fluconazole plus placebo c(n = 5)	P-value
Vaginal discharge associated with at least one of the symptoms (itching and burning vaginal feeling, dyspareunia and dysuria)	2 (18·2)	2 (40·0)	0·547
Presence of *Candida* by culture method	2 (18·2)	4 (80·0)	0·036
Positive 'Whiff' test	0 (0·0)	1 (20·0)	0·313
Vaginal pH >4·5	1 (9·1)	0 (0·0)	>0·999
Nugent BV score	0 (0·0)	1 (20·0)	0·313

Results are expressed as absolute numbers and percentages respectively*.
*For proportions Fisher's exact two-tailed test was used

treated with fluconazole and placebo ($P < 0·05$) (Table 2). No statistical difference between both groups for there was positive 'Whiff' test, vaginal pH higher than 4·5 and Nugent BV Score ($P > 0·05$).

At the end of the treatment, we performed an analysis of subjects who had a history of recurrent VVC. Of these, more subjects in the placebo group were positive for yeasts than in the probiotic group (80·0% and 18·2% respectively) as determined by culture method ($P < 0·05$) (Table 3). However, there was no statistical difference in clinical cure rates (remission of symptoms) when both groups were assessed ($P > 0·05$) (Table 3).

Of the six subjects diagnosed with *C.* non-*albicans* VVC, cure rates did not differ between the groups ($P > 0·05$). Two subjects in the fluconazole and probiotic group reported an increase in appetite, one had an episode of headache and another reported an occurrence of light stool, none of which could conclusively be attributed to the probiotic.

Discussion

In the present study, supplementation of a single dose of fluconazole with 28 days of probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 resulted in superior clinical and microbiological cure of VVC. This is the first report of a probiotic augmenting the efficacy of an anti-fungal agent. The use of oral azoles is generally effective against VVC (Sobel 2007), but in the present population, symptoms and signs of disease remained at 4 weeks in over one-third of subjects given the drug plus placebo. It is not possible to say that the present findings are unique to the two strains used without a comparative study with another probiotic. Likewise, the key mechanisms involved in this study have not been uncovered, thus the role of metabolic by-products of the probiotics may or may not have influenced the outcome. However, we would not expect dead GR-1 and RC-14 to be as effective, as they would not multiply in the gut, not produce metabolites and only function by gut competition (not relevant to the vagina) or lipoteichoic acid immune modulation via the small intestine. Rather, we believe the reasons why these probiotic lactobacilli reduced this failure rate to under 11% could involve small intestine, colonic and vaginal immune modulation (Lorea Baroja *et al.* 2007), inhibition of the growth of *C. albicans* in the vagina (Reid *et al.* 2006) and/or reduced ascension of yeast from the rectum to vagina (Reid *et al.* 2003).

According to a study conducted by Ćorić *et al.* (2006), 14 days after the treatment of patients with VVC using either a single dose of 150 mg fluconazole or 200 mg clotrimazole as a 3-day intravaginal regimen, *Candida* micro-organisms were found in 27·0% and 31·0% of the patients respectively. In our study, 38·5% of the patients treated with a single dose of 150 mg fluconazole and placebo capsules were still positive for the micro-organisms in the follow-up performed 4 weeks after the initial treatment. In contrast, only 10·3% of the patients concomitantly treated with fluconazole and probiotics showed the presence of *Candida* spp. This, in addition to the remission of symptoms and signs of infection, further indicates a successful cure of the infection by probiotic supplementation in most individuals. The high prevalence of *C. albicans* (over 84%) compared favourably with those reported by other sites over a 5-year period (Paulitsch *et al.* 2006).

To assess the adverse effects of probiotics, monitoring is required, especially, if invasive procedures are performed on patients or the subjects are at a high risk of bacteremia because of, for example, a leaky bowel. However, in the present study, all subjects were otherwise healthy and at low risk of complications, and indeed, there were only rare reports of mild adverse effects that could not be definitively associated with the probiotics.

In conclusion, our clinical study showed that probiotic lactobacilli augmented anti-fungal treatment of VVC. This finding has implications for extending the longevity and effectiveness of anti-infective pharmaceutical agents, especially at a time when so few new drugs are in the pipeline and resistant organisms are increasingly emerging.

Acknowledgements

R.C.R. Martinez is grateful to São Paulo State Foundation for Support of Science (FAPESP, process no. 04/14580-0) and to the Brazilian Post Graduate Federal Agency (CAPES, process no. 6159/06-2) for supporting his PhD studies in Brazil and Canada respectively. The authors acknowledge FAPESP for financial support (process no. 06/06595-2). The authors are grateful to Prof. Augusto C. C. Spadaro, PhD and Prof. Sérgio de Albuquerque, PhD, from FCFRP–USP (Brazil) for administrative support throughout this study, Chr. Hansen for providing the capsules for this study and Dr Andrew W. Bruce for his input to the design. The authors are thankful for the collaboration of the excellent team of physicians and nurses and also for the study volunteers recruited at public health centres in the city of Ribeirão Preto, São Paulo State, Brazil. Assistance from a grant from Natural Sciences and Engineering Research Council of Canada (NSERC) is appreciated.

Conflict of Interest Statement

G. Reid holds some patents associated with lactobacilli. However, his input was in protocol design, logistics, student supervision and assistance with the manuscript, not in the accumulation of data, and he was blinded to the results until after the code was broken and findings acquired.

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