Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), *Lactobacillus rhamnosus* GR-1, and *Lactobacillus reuteri* RC-14: a randomized, double-blind, placebo-controlled trial

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Abstract: Bacterial vaginosis (BV) is the most prevalent vaginal infection worldwide and is characterized by depletion of the indigenous lactobacilli. Antimicrobial therapy is often ineffective. We hypothesized that probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 might provide an adjunct to antimicrobial treatment and improve cure rates. Sixty-four Brazilian women diagnosed with BV were randomly assigned to receive a single dose of tinidazole (2 g) supplemented with either 2 placebo capsules or 2 capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 every morning for the following 4 weeks. At the end of treatment (day 28), the probiotic group had a significantly higher cure rate of BV (87.5%) than the placebo group (50.0%) ($p = 0.001$). In addition, according to the Gram-stain Nugent score, more women were assessed with "normal" vaginal microbiota in the probiotic group (75.0% vs. 34.4% in the placebo group; $p = 0.011$). This study shows that probiotic lactobacilli can provide benefits to women being treated with antibiotics for an infectious condition.

Key words: bacterial vaginosis, tinidazole, probiotic, *Lactobacillus rhamnosus* GR-1, *Lactobacillus reuteri* RC-14.

Résumé : La vaginite bactérienne (VB) est l'infection vaginale la plus commune dans le monde et elle est caractérisée par une déplétion des lactobacilles indigènes. La thérapie antimicrobienne est souvent inefficace. Nous émettons l'hypothèse que les probiotiques *Lactobacillus rhamnosus* GR-1et *Lactobacillus reuteri* RC-14 puissent fournir un adjuvant au traitement antimicrobien et améliorer le taux de guérison. Soixante-quatre Brésiliennes souffrant de VB ont été choisies de façon aléatoire pour recevoir une seule dose de tinidazole (2 g) et 2 capsules de placebo ou 2 capsules contenant *L. rhamnosus* GR-1 et *L. reuteri* RC-14 à chaque matin pendant les 4 semaines suivantes. À la fin du traitement ($28^{\text{ième}}$ jour), le groupe avec probiotiques affichait un taux de guérison significativement plus élevé (87.5 %) comparativement au groupe placebo (50.0 %)($p = 0.0001$). Aussi, selon le score de Nugent après coloration de Gram, plus de femmes ont été considérées porteuses d'une flore vaginale microbiotique normale dans le groupe avec probiotiques (75.0 % versus 34.4 % chez le groupe placebo; $p = 0.011$). Cette étude démontre que les lactobacilles probiotiques peuvent être bénéfiques aux femmes traitées avec des antibiotiques pour une condition infectieuse.

Mots-clés : vaginite bactérienne, tinidazole, probiotique, *Lactobacillus rhamnosus* GR-1, *Lactobacillus reuteri* RC-14.

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Introduction

Bacterial vaginosis (BV) is a condition characterized by both a significant reduction in vaginal lactobacilli, replaced by facultative aerobic and anaerobic microorganisms such as *Prevotella* spp., *Gardnerella vaginalis*, *Mobilluncus* spp., and *Atopobium* spp. among others, and a pH elevated above 4.5 (Burton et al. 2004; Wilks et al. 2004). Microorganisms associated with BV have the potential to produce inflammatory mediators, including phospholipases, leading to increased production of prostaglandins and risk of preterm labor (McGregor et al. 1991, 1992). The condition also increases the risk of pelvic and sexually transmitted infections (Amsel et al. 1983; Morris et al. 2001).

BV accounts for up to 40%–50% of cases with symptomatic vaginal discharge (O'Brien and Serwint 2008), and

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the prevalence of the condition during pregnancy may range from 12% to 55%, according to different diagnostic methods employed (Hogan et al. 2007). At any given time, an estimated 29% of women have an aberrant vaginal microbiota indicative of this condition, even if they are not all symptomatic (Allsworth and Peipert 2007). Recurrent BV, defined as 4 or more episodes of the disease within a period of 1 year, is quite common, and indeed between 30% and 58% of women experience recurrence of symptomatic BV over the course of a 12 month period after recommended oral therapy, which is also associated with recurrence of abnormal vaginal flora (Bradshaw et al. 2006*a*, 2006*b*; Myer et al. 2006).

Amsel et al. (1983) suggested the confirmation of BV by at least 3 of the following findings: (*i*) vaginal pH > 4.5, (*ii*) aqueous white vaginal discharge, (*iii*) fishy odor in the "Whiff" test, and (*iv*) presence of "clue cells." Nugent et al. (1991) proposed use of a scoring system for Gram-stained vaginal exudates, based on the quantity and morphology of Gram-positive and Gram-negative organisms present: "normal" (0–3), "intermediate" (4–6), and "BV" (7–10).

Relatively few options are available for the management of BV, and treatments recommended by the Centers for Disease Control and Prevention in the United States include metronidazole in tablet (500 mg, twice daily for 7 days) or gel (0.75%, once daily for 5 days) forms, and clindamycin 2.0% in vaginal cream for 7 days (Centers for Disease Control and Prevention (CDC) 2002). However, these therapeutic regimens may exhibit cure rates of 60% or even lower when 4 week cure rates are considered (Larsson and Forsum 2005).

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). A previous study reported that adjunctive use of metronidazole and orally administered probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 could improve the cure of BV (Anukam et al. 2006*a*). Other investigators found variable results with the use of *Lactobacillus* in the treatment of BV, and limited documentation of strain-specific effects currently prevents probiotics being recommended for the management of the condition (Barrons and Tassone 2008).

The present study was designed to assess whether probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 could be effective when combined with tinidazole for the treatment of BV. Tinidazole was previously shown to reduce the rates of prematurity and perinatal complications among Brazilian pregnant women with BV (Camargo et al. 2005). Recently, tinidazole has been approved by the United States Food and Drug Administration (FDA) for BV treatment, and it is under consideration as an alternative to metronidazole (Nailor and Sobel 2007).

Materials and methods

Probiotic organisms

Probiotic strains *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were prepared under FDA good manufacturing practices by Chr. Hansen (Horsholm, Denmark). The bacteria were dried and placed in gelatin capsules containing 1×10^9 CFU viable cells of each strain. Gelatin capsules containing cellulose and magnesium stearate were used as placebo.

Human subjects, randomization, and protocol

Subjects were recruited and examined by gynecologists from 4 local health centers affiliated with Universidade de São Paulo (USP): (*i*) Centro de Saúde Escola (CSE) da Faculdade de Medicina de Ribeirão Preto (FMRP), (*ii*) Hospital das Clínicas (HC)-FMRP, (*iii*) Sistema Integrado de Saúde (SIS), and (*iv*) Centro de Saúde Vila Lobato (CSVL)-FMRP, Brazil. Each subject signed an informed consent under a protocol (Protocol No. 0146) approved by the Ethics Review Board at the CSE-FMRP-USP. This study was registered online at Comissão Nacional de Ética em Pesquisa (CONEP; Document No. 070202), Brazil.

Subjects were voluntarily enrolled in the study if they were diagnosed with BV according to the Amsel criteria (Amsel et al. 1983) and Nugent scoring (Nugent et al. 1991). This included analysis of vaginal discharge, vaginal pH (pH indicator strip: Acilit pH 0–6; Merck, Germany), and a "Whiff" test, following collection of 3 vaginal samples (using 2 swabs and an Ayres spatula), plus a vaginal smear that was Gram-stained and visualized under oil immersion (1000× magnification) and interpreted on a 10-point scale based on the presence or absence of *Lactobacillus* morphotypes, *G. vaginalis*, *Mobilluncus* spp., and other microorganisms according to Nugent et al. (1991).

Subjects were not included if they were immunosuppressed, diagnosed with concomitant vulvovaginal candidiasis and (or) trichomoniasis, were using systemic or intravaginal antibiotic or antifungal agents currently or within the past 2 weeks of the appointment, were in menses, or were allergic to imidazoles.

Identical vials containing probiotics and placebo were received from the manufacturer in separated boxes with 2 different colors and they were random numbered, by staff not participating in the study, in the laboratory at Universidade de São Paulo, Brazil. Investigators were blinded to the randomization code until all data were analyzed. Equal parts of probiotics and placebo vials were placed in plastic bags and given to investigators to take to their health centers and randomly distribute to patients enrolled in the study. The subjects were treated on a firstcome basis with a single dose of tinidazole (2 g) plus either 2 oral capsules of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or 2 placebo capsules taken daily in the morning for 28 days, starting on the first day of tinidazole use. Assuming an anticipated cure rate of 60% in the control group arm, a minimum of 25 subjects per group was required to detect an increase in the cure rate as compared with 90% in the group supplemented with the probiotic microorganisms, assessed at the 2-sided 5% level of significance with 80% power.

After 4 weeks of treatment, the same procedure adopted during the first appointment with the gynecologist was performed. The presence of any symptoms and signs (vaginal discharge, fishy odor, or burning and itching vaginal feeling) or possible side effect related to the drug and (or) probiotic were recorded. The investigators remained blinded to the study codes until all analyses had been completed. All subjects stated adherence to the protocol, as a measure of compliance, and many returned the empty vials.

Statistical analysis

To compare the initial characteristics of patients enrolled

Table 1. Demographic and behavioural characteristics, as well as infection symptoms and signs, and results of laboratory determinations on vaginal samples obtained from Brazilian women diagnosed with bacterial vaginosis.

Observation	Probiotic group (n = 32)*	Placebo group (n = 32)*	p
Mean age (range) in years	30.0±10.9 (16–51)	30.3±10.7 (16–50)	0.912†
Use of contraceptive methods	21 (65.6)	19 (59.4)	0.606‡
Regular menses	19 (59.4)	20 (58.8)	0.798‡
Recurrent BV (self-reported)	8 (25.0)	7 (21.9)	0.768‡
Positive Amsel test	32 (100.0)	32 (100.0)	—
Vaginal pH > 4.5	31 (96.9)	29 (90.6)	0.613§
White and (or) homogeneous and (or) aqueous vaginal discharge	32 (100.0)	32 (100.0)	—
Positive "Whiff" test	32 (100.0)	31 (96.9)	>0.999§
Bad vaginal odor (self-reported)	30 (93.8)	28 (87.5)	0.672§
BV status assessed by the Nugent score	31 (96.9)	32 (100.0)	>0.999§

Note: Each study group consisted of 32 patients. Laboratory samples were collected at the first gynecologic visit pre-treatment. BV, bacterial vaginosis; probiotic group, group randomized to orally administered *Lactobacillus rhamnosus* GR-1 plus *Lactobacillus reuteri* RC-14, 2 capsules once daily for 4 weeks, as adjunct to single-dose tinidazole (2 g) treatment; placebo group, group randomized to orally adminstered placebo, 2 capsules once daily for 4 weeks, after single-dose tinidazole (2 g) treatment.

*Results are expressed as absolute numbers with percentages in parentheses.

†Values were obtained using the t test.

‡Values were obtained using the χ^2 test.

§Values were obtained using Fisher's exact 2-tailed test.

in the probiotic and placebo groups as well as the outcomes obtained between both groups, the t test, the χ^2 test, and Fisher's exact 2-tailed test were used. In all cases, a significance level of 5% was adopted. SAS software, version 9.1 (SAS Institute Inc., Cary, North Carolina) was used to perform the tests.

Results

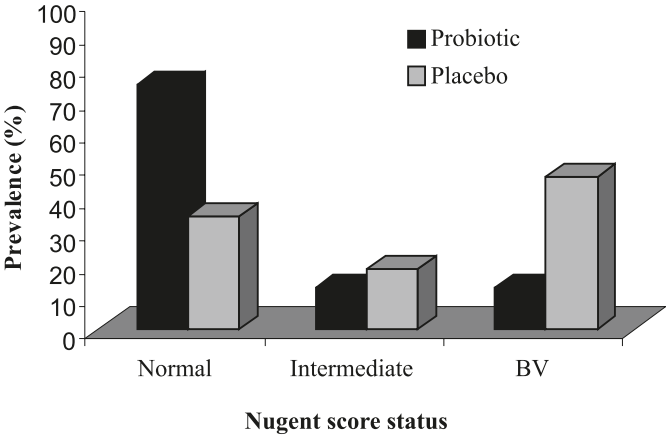
The demographic and behavioral characteristics, as well as symptoms and signs of infection assessed during the initial appointment with the gynecologist, and the results of laboratory determinations obtained from 64 subjects enrolled in the 2 groups of patients are summarized in Table 1. Subjects who self-reported as suffering from recurrent BV, were equally distributed in the 2 groups: 8 (25.0%) and 7 (21.9%) in the probiotic and placebo groups, respectively.

On the 28th day of the study, more subjects in the probiotic group than in the placebo group showed normal vaginal smears according to the Nugent score (p = 0.011) (Fig. 1) and Amsel test (p = 0.001) (Table 2). This translated to a cure rate of 87.5% for tinidazole plus lactobacilli, vs. 50% with tinidazole and placebo ($p < 0.05$). The vaginal smears in the placebo group showed a higher presence of Gram-variable cocci-rods and curved Gram-negative rods indicative of BV ($p < 0.05$) (Fig. 1). In addition, 4 subjects (12.5%) in the placebo group and one (3.1%) in the probotic group developed vulvovaginal candidiasis post-treatment, although this was not statistically significant (p = 0.474).

The subjects who had self-reported recurrent BV were evaluated according to the Amsel test and the Nugent score, and it was found that 75.0% of patients in the probiotic group were cured in comparison with 57.1% for the placebo group ($p > 0.05$).

One subject (3.1%) in the tinidazole plus probiotic group reported a persistent headache episode that was not believed to be associated with the probiotic treatment.

Fig. 1. Prevalence (%) of women assessed with normal, intermediate, or bacterial vaginosis (BV) microbiota, evaluated by the Nugent score after treatment with a single dose of tinidazole (2 g) supplemented with either 2 daily probiotic (*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14) or placebo capsules, every morning, for 4 weeks. Each group consisted of 32 patients. More patients in the probiotic group (75.0%) showed normal vaginal microbiota when compared with those in the placebo group (34.4%) (p = 0.011). In addition, more patients in the placebo group (46.9%) showed BV vaginal microbiota compared with those allocated to the probiotic group (12.5%) (p = 0.003).



Discussion

According to Marrazzo et al. (2006), given the high prevalence of BV, there is an urgent need to develop products that effectively treat the condition and prevent its recurrence.

Table 2. Comparison of the effects of single-dose tinidazole (2 g) with 28 days of once-daily oral intake of either placebo or *Lactobacillus rhamnosus* GR-1 plus *Lactobacillus reuteri* RC-14 capsules for the treatment of bacterial vaginosis in women in Brazil.

Outcome	Tinidazole plus lactobacilli (n = 32)*	Tinidazole plus placebo (n = 32)*	$p^{\dagger}$
Positive Amsel test	4 (12.5)	16 (50.0)	0.001
Vaginal pH > 4.5	9 (28.1)	17 (53.1)	0.042
White and (or) homogeneous and (or) aqueous vaginal discharge	6 (18.8)	18 (56.3)	0.002
Positive "Whiff" test	6 (18.1)	18 (56.3)	0.002
Bad vaginal odor (self-reported)	3 (9.4)	9 (28.1)	0.055

*Results are expressed as absolute numbers with pecentages in parentheses.
†A χ^2 test was used, except for counts <5 when Fisher's exact 2-tailed test was performed.

Although many patients do not have the classic discharge and odor associated with BV (Klebanoff et al. 2004), the condition has an adverse effect on quality of life (Karasz and Anderson 2003), and those with recurrences often pursue alternative treatment strategies. The current findings that probiotic lactobacilli can improve the cure rate of antibiotics provides support for complementary therapy for BV.

Tinidazole, a second-generation nitroimidazole derivative with a structure and activity similar to metronidazole, was used instead of 7 days of metronidazole for several reasons: (*i*) better compliance of tinidazole as a single-dose therapy; (*ii*) oral tinidazole achieves higher serum peaks and reproductive tissue concentrations, shows enhanced tolerance, has fewer side effects, and has reduced toxicity and a longer half-life (Bagnoli 1994); and (*iii*) tinidazole is more lipid soluble than metronidazole, which allows it to penetrate cell membranes more effectively (Jokipii et al. 1977). The finding that only 1 subject who used the antibiotic and probiotic capsules stated an adverse effect, an episode of headache, indicates good tolerance in this study. The actual cure rate in the tinidazole and placebo group was relatively low, yet still higher than a previous study of 235 patients (Livengood et al. 2007) in which the cure rate was 36.8% with 1 g of tinidazole once daily for 5 days, and 27.4% with 2 g once daily for 2 days. Thus, the cure rate of 87.5% with tinidazole plus probiotics, in line with a previous study using metronidazole and probiotics, shows the extent to which probiotics enhanced the outcome (Anukam et al. 2006*a*).

Not all lactobacilli products are effective, nor are others so far that have been given orally. Eriksson et al. (2005) reported no significant difference in prevention of recurrent BV when subjects were treated with clindamycin suppositories for 3 days followed by 5 days of treatment with randomized tampons containing freeze-dried *Lactobacillus* strains (*L. fermentum*, *L. casei-rhamnosus*, and *L. gasserii*) or placebo (56% and 62% cure rates, respectively). In 2 studies, intravaginal administration of lactobacilli had some effect (Drago et al. 2007; Larsson et al. 2008). In the Drago study (Drago et al. 2007), an open pilot format was used on 40 BV cases, and it was found that treatment for 6 days with a douche containing *L. acidophilus* led to improved Nugent scores that remained low during the follow-up period (20 days after the last treatment) for almost all of the patients. The authors also observed decreases in vaginal pH to less than 4.5 in 30 of 40 subjects. In the Larsson study (Larsson et al. 2008), vaginal clindamycin therapy followed by intravaginal insertion of freeze-dried lactobacilli capsules for 10 days during 3 menstrual cycles did not improve the efficacy of BV therapy during the first month of treatment, but for women initially cured, it lengthened the time to relapse significantly at 6 month follow-up (Larsson et al. 2008). The fact that 5 days of intravaginal use of *Lactobacillus* GR-1 and RC14 strains cured BV better than metronidazole vaginal gel, in another study (Anukam et al. 2006*b*), emphasizes the importance of selecting the right probiotic products.

Many studies have evaluated the cure rate of BV after 1 week of treatment, but according to Larsson (1995), 1 week follow-up is too short a time for meaningful interpretation of individual patient data, and a better time point is 1 month, as was used here.

In our study, 25.0% and 21.9% of patients from the probiotic and placebo groups, respectively, self-reported as having recurrent BV. Although this is not a fully reliable method to identify patients with recurrence, we found higher cure rates of the infection in the probiotic group (75%), compared with the placebo group (57.1%). Usually, this hard-to-treat group of patients requires prolonged use of oral or topical agents such as imidazole (CDC 2002). In the present study, there was no 3 month follow-up, so no conclusions can be drawn with respect to preventing recurrences.

In terms of understanding how lactobacilli might intervene against BV, Saunders et al. (2007) showed that *L. crispatus* 33820, *L. iners* AB-1, *L. rhamnosus* GR-1, and especially *L. reuteri* RC-14 could displace *G. vaginalis* biofilms in vitro. This was not due to pH, which remained between 4.7 and 5.1 in all experiments, nor to hydrogen peroxide, which is produced in low amounts by this strain and in high amounts by *L. crispatus* 33820. The authors suggested that biosurfactants produced by *L. reuteri* RC-14 (Velraeds et al. 1996) may play a role in displacement, while production of bacteriocins and signaling molecules (Reid 2001; Laughton et al. 2006) may have affected viability and pathogen growth. However, more studies are required to know how the strains functioned here.

In summary, our results show that the conjoint use of single-dose tinidazole (2 g) with orally administered probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 augmented cure of Brazilian patients diagnosed with BV. These findings raise the possibility of combining pharmaceutical agents with probiotics as a means to better manage common infections, perhaps extend the longevity of drugs whose efficacy

is waning through bacterial resistance, and reduce the number of women requiring long-term prophylaxis to prevent recurrences.

Potential conflicts of interest

G. Reid holds 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 like all authors, he was blinded to the results until after the code was broken and the findings acquired.

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