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Article type : Original Article

Randomized clinical trial in women with Recurrent Vulvovaginal Candidiasis: efficacy of probiotics and lactoferrin as maintenance treatment.

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Running head: Lactobacilli and lactoferrin for VVC treatment.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/myc.12883

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Keywords: *Lactobacillus acidophilus*; *Lactobacillus rhamnosus*; Bovine lactoferrin; Vulvovaginal infections; *Candida* spp.; Respecta®

Summary

Background. Vulvovaginal candidiasis (VVC) is a recurrent vaginal condition in childbearing women.

Objectives. The aim of this study was to assess the efficacy of an oral formulation containing *Lactobacillus acidophilus* GLA-14, *Lactobacillus rhamnosus* HN001 and bovine lactoferrin on symptoms and recurrence of VVC as adjuvant therapy to topical clotrimazole.

Patients/Methods. Forty-eight women positive for *C. albicans*, symptoms of VVC and documented history of recurrences were randomized into 2 groups receiving verum or placebo (2 capsules/day for 5 days followed by 1 capsule/day for additional 10 days) as adjuvant treatment to clotrimazole (induction phase) followed by a maintenance cycle of 6 months (1 capsule/day verum or placebo for 10 consecutive days each month). Symptoms, overall cure rate and recurrence rate were assessed.

Results. After clotrimazole therapy, a significant improvement of symptoms was shown in both groups. However, only women treated with probiotics and lactoferrin showed a significant improvement of itching and discharge at 3 and 6 months. During the six-month follow-up, recurrences were significantly less in the intervention group vs placebo (33.3% vs 91.7% after 3 months and 29.2% vs 100% after 6 months).

Conclusions. The results show that the investigated lactobacilli mixture in combination with lactoferrin represents a safe and effective adjuvant approach for reducing symptoms and recurrences of RVVC.

1. Introduction

Vaginitis is a common gynecologic disorder that is responsible for 10 million office visits to physicians each year. In particular, fungal infection of the vulva and the vagina is estimated to be the second most common cause of inflammation after bacterial vaginosis¹. About 75% of women during their reproductive age have at least one episode of vulvovaginal candidiasis (VVC)² and approximately half of them have two or more episodes. The most common pathogen is *Candida albicans*, which is isolated in 85% to 90% of all cases³. Asymptomatic colonization with *Candida* spp. is also common. It can be found in about one-third of women without any symptoms and was identified in 70% of women during a 1-year observation period⁴. Recurrent vulvovaginal candidiasis (RVVC) is defined as the 4 or more episodes of symptomatic VVC within one year⁵. RVVC is less common than acute form, affecting up to 9% of women of reproductive age but can greatly affect the quality of life of women, causing symptoms such as itching and soreness of the vulva, dyspareunia, dysuria and the classic ‘cottage cheese-like’ discharge⁵. It is most commonly caused by *Candida albicans*, but other non-*albicans* species such as *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis*, although less frequent, are more commonly associated with recurrence^{6,7}. An increased rate of vaginal colonization represents a phase of susceptibility to RVVC and causes may be genetic, biologic, or behavioral: diabetes, atopy, antibiotic or corticosteroids use, vaginal douching, orogenital sex etc. Not only, but also some primary or idiopathic RVVC defines women in whom secondary precipitating events or triggering factors are not apparent and hence genetic factors are likely to play a dominant or exclusive mechanism⁸. It is proposed that both RVVC and VVC involve similar immunopathologies but that the triggers occur with greater sensitivity in individuals with RVVC. Women with RVVC (approximately 150 million worldwide) result in idiopathic chronic episodes of vaginal irritation that require antifungal maintenance therapy (e.g., azoles) to partially control symptoms⁹. Although these

treatments are typically effective at reducing organism burden and symptoms, the static function of azole activity and fungal recalcitrance to clearance are key factors resulting in recurrence. The most widely used form of treatment involves an intense induction therapy with antifungals, followed by an extended period of maintenance therapy for some months¹⁰⁻¹². Despite this is being generally accepted as standard treatment, many variations exist including different therapeutic agents and durations^{13,14}.

Current evidence suggests that in the case of induction and maintenance therapy with fluconazole, approximately 50% of patients experience relapse after six months^{15,16}. Studies evaluating the effectiveness of probiotics in preventing RVVC have shown conflicting results. A report by De Seta et al.¹⁷ evaluated the effectiveness of local *Lactobacillus plantarum* P17630. One group of patients received the standard treatment with local clotrimazole for 3 days and the other group had additional probiotic capsules applied intravaginally for 6 days and then once weekly for 4 weeks. They concluded that local *Lactobacillus plantarum* P17630 offers potential benefit for resolution of vaginal discomfort but no data are showed for the recurrence rate. Results from a review study¹⁸ support the effectiveness of both oral and local lactobacilli in preventing new episodes of vaginitis, particularly *Lactobacillus rhamnosus* GR-1, *Lactobacillus fermentum* RC- 14, and *Lactobacillus acidophilus*, whereas other studies did not prove the effectiveness of lactobacilli (Falagas et al. 2006).

De Seta et al.¹⁹ published additional evidence supporting the use of specific lactobacilli able to reduced significantly the recurrence of *Candida* spp. infection. Until today, published studies show several limitations including the small sample size, absence of placebo group, high heterogeneity of probiotic strains, dose and treatment duration¹⁸. It should be emphasized that the various probiotic strains have different properties and different effects on *Candida* spp.; thus, results from studies testing one strain should not be extrapolated to other

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strains. Consequently, it is difficult to draw reliable conclusions from the existing studies. Prospective, randomized, double-blind, placebo-controlled trials able to assess vaginal colonization and clinical outcomes are very few. A recent Cochrane review compared conventional antifungal drugs used as single treatment to probiotics as adjuvant therapy for enhancing short-term (5-10 days) clinical and mycological cure and relapse or recurrence episodes over time. Adjunctive treatment does not seem to influence the rate of long-term (within one to three months) clinical cure, long-term mycological cure, serious and non-serious side events. Up to the present, due to the low quality of data available, there is insufficient evidence for the use of probiotics either as adjuvants to conventional antifungal medicines or used alone for the treatment of VVC in non-pregnant women²⁰.

In addition to lactobacilli, among a variety of natural substances, lactoferrin seems to be one of the most interesting compound for reducing the risk of vaginal candida infection. Lactoferrin is an iron-binding glycoprotein naturally present in mammals' secretions including milk and cervical mucus. It is stored in the secondary granules of neutrophils from where it is released in inflamed sites^{21,22}. *In vitro* evidence showed that both human and bovine lactoferrin is able to inhibit the growth of *C. albicans* and *C. glabrata* as well as the production of inflammatory cytokines^{23,24}. Recent findings showed that lactoferrin was able to improve the biofilm production of two lactobacilli strains cultured on HeLa cell monolayer thus enhancing their protective action against pathogen bacteria adhesion to genital cells²⁵.

The aim of the current study was to evaluate clinical cure rate, and recurrences of infection at 1, 3 and 6 months in women with RVVC treated with oral probiotics in combination with bovine lactoferrin as adjuvant therapy after an induction phase with clotrimazole.

2. Patients and Methods

2.1 Study design and general procedures

The current double-blinded, prospective, randomized, clinical trial was carried out in accordance with Good Clinical Practices and the World Medical Association (WMA) Declaration of Helsinki regarding the Ethical Principles for Medical Research Involving Human Subjects adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and its following amendments. The study protocol was approved by the National Commission on Bioethics of Drugs and Medical Devices (Bucharest, approval number 2DM/26.01.2016 March, 14th 2016).

Forty-eight women with positive cultures for *Candida* spp., symptomatic acute episode of VVC and with documented anamnestic history of recurrences confirmed by culture analysis were enrolled according to the inclusion and exclusion criteria (table 1). Women were informed about the study protocol, procedures, investigational product and potential risks of treatment. After signing the informed consent, women were randomly divided into 2 groups (24 subjects per group) according to a computer randomized 1:1 scheme to receive either verum or placebo as supplementary treatment to antifungal drug with an induction scheme with topical clotrimazole. Figure 1 shows the study flow chart.

During the screening visit before treatment allocation, the gynecologist collected a vaginal swab from each woman by careful rotation of sterile cotton swab against sidewalls in the upper third area of the vagina for performing the culture analysis (culture for bacteria, yeast and trichomonas) for diagnostic confirmation of *Candida* spp. and gram stain (Nugent score) to exclude bacterial vaginosis. Six visits were scheduled during the study at participating gynecological centers. During the first visit (T0) at baseline, patient medical history was recorded, informed consent was signed and women were allocated into the two study arms.

Controls were performed 1 week (T1), 2 weeks (T2), 1 month (T3), 3 months (T4) and 6 months (T5) after initial antifungal treatment and were aimed to assess the efficacy and safety of the investigational products. The symptoms evaluated (itching and vaginal discharge) were self-reported by the patients as present or absent since the previous gynecologic visit.

The evaluation of efficacy was based on clinical overall cure rate defined as the ratio between the number of women without vaginal discharge or itching and negative culture and all women present in the treatment group. In addition, recurrence rate was calculated for both groups as ratio between the number of women experiencing at least one symptom during the 6 months follow-up period, confirmed by culture, and all women present in the treatment group. Safety was assessed by recording all side effects (i.e. adverse events, serious adverse events, and suspected unexpected serious adverse reactions). An adverse event was considered severe if it was fatal, life threatening, required hospitalization, or led to permanent injury.

2.2 Treatment

The investigational product, Respecta[®], is registered and manufactured as a medical device class IIa by Giellepi S.p.A. Health Science (Lissone, MB, Italy). It contains a proprietary lactobacilli mixture (5×10^9 CFU per capsule) including *Lactobacillus acidophilus* GLA-14 (BCCM/LMG Bacteria Collection, LMG S-29159) and *Lactobacillus rhamnosus* HN001 (American Tissue Culture Collection, ATCC Number: SD5675) in combination with bovine lactoferrin RCX[™] (50 mg). Placebo was an identical capsule containing the inactive ingredient maltodextrin (100 mg); both products were produced with the same excipients.

Treatment consisted in 2 different phases; during the induction phase, all enrolled women were treated with standard antifungal therapy (clotrimazole 100 mg daily for 7 days) and randomly assigned to one of two study arms receiving either verum (Respecta[®]) or placebo (2 capsules/day for 5 days followed by further 10 consecutive days at the dosage of 1 capsule/day) by oral intake (acute treatment). Respecta[®] or placebo were administered simultaneously to the antifungal drug. During the maintenance phase (6 months following the induction phase), all recruited women ingested 1 capsule of Respecta[®] or placebo per day, for 10 consecutive days per month in premenstrual phase (prophylactic treatment). This timeline was related to the consideration that the recurrence, typically, occurs in premenstrual or luteal phase. In fact, during premenstrual phase, for some hormonal and immunological reasons (high estrogen, increasing progesterone, higher level of inflammatory cytokines), the vagina become more vulnerable to the pathogens. For these reasons, we decide to perform maintenance cycle with probiotics during a sort of “window period” at high risk of recurrences^{26,27}.

Compliance was determined by assessing returned packages.

2.3 Statistical analysis

Descriptive statistics was used for the demographics and clinical evaluations. Differences between the study arms were assessed by Chi-square test. The statistical significance was set at $P < 0.05$.

3. Results

3.1 Demographic characteristics

All recruited women completed the study and compliance was 100% for all participants. Table 2 shows demographic characteristics of women from both study arms. No significant differences were observed.

3.2 Safety evaluation

No woman experienced adverse events during the study period.

3.3 Clinical Cure Rate (itching and vaginal discharge)

Itching

After the clotrimazole treatment (T1), a significant improvement of itching was shown in both placebo and Respecta[®] arms. However, only this latter improved itching significantly during the follow-up period at T4 (3 months) and T5 (6 months). In particular, women without itching in the intervention group in comparison to placebo were 70.8% vs 8.3% at T4 and 83.3% vs 0% at T5, respectively ($P < 0.01$ Respecta[®] vs placebo). Study results are shown in figure 2.

Vaginal discharge

After one week of treatment (T1), no women showed vaginal discharge in both arms of the study probably related to the antifungal therapy effects. Symptoms appeared in a few women from each of the groups within one week after the end of drug administration and no

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significant difference was recorded between verum or placebo at T2 and T3. On the contrary, it was only in the intervention group that vaginal discharge improved significantly during the follow-up period at T4 and T5. In particular, women treated with Respecta[®] remained vaginal discharge free in 66.7% of cases (vs 8.3% in placebo group) at T4 and in 70.8% of cases (vs 20.8% in placebo group) at T5 ($P < 0.01$ Respecta[®] vs placebo for both T4 and T5). Study results are shown in figure 3.

3.4 Overall Cure Rate

The investigational product during the maintenance phase improved the clinical overall cure rate showing the absence of any symptoms (neither itching nor vaginal discharge) and of yeasts indicated by culture from swab, with a significant difference between the two study arms at T4 and T5. In particular, the overall cure rate in the intervention group vs placebo was 66.7% vs 8.3% at T4 and 70.8% vs 0% at end of follow-up (T5; $**P < 0.01$ Respecta[®] vs placebo). Results are shown in figure 4.

3.5 Recurrence Rate

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Significantly fewer recurrences were found in women treated with the investigational product during the 6-months follow-up in comparison to those who took the placebo. In particular, the recurrence rates, defined as presence of at least one VVC symptom confirmed by vaginal culture, were significantly lower in the intervention group than in placebo group during the maintenance phase, being 33.3% at T4 and 29.2% at T5 in the Respecta[®] group and 91.7% at T4 and 100% at T5 in the placebo arm ($**P < 0.01$ Respecta[®] vs placebo). Results are shown in figure 5.

4. Discussion

Cases of RVVC are hard to manage and require prolonged treatment¹¹. International guidelines for the treatment of RVVC are consistent. However, the suggested treatments are not particularly effective and a majority of women relapse following several types of treatment and the question of duration of therapy remains unclear. Despite most guidelines agreeing on six months of oral anti-fungal therapy as the appropriate treatment, the results from this regimen are disappointing. A longer course may be appropriate^{8,12}, but this is not yet supported by large-scale studies. Therefore, further research is needed to address this issue. Generally, several factors have been shown to contribute to the acquisition and/or induction of a symptomatic recurrence or chronicity of vulvar candidiasis⁸. These include intestinal reservoir, persistent vaginal yeasts, sexual transmission²⁸, reduced protective vaginal factors²⁹, acquired acute hypersensitivity reaction³⁰ and host genetic susceptibility^{31,32}. Most existing regimens in use for recurrent or chronic fungal vulvovaginal infections are empirical and not evidence based³³. In 2004, data published from Sobel et al.¹⁵ concluded that long term weekly treatment with fluconazole could reduce the rate of recurrence of symptomatic vulvovaginal candidiasis.

Principles of therapy include induction therapy, followed by a maintenance therapy for 6 months³⁴. Therapy interruption may result in relapse in about 50% of patients. Alternative therapies should be considered for recalcitrant cases or with the aim to reduce azole use. In fact, a growing number of women are troubled by the high prevalence and recurrence rates of VVC and, although anti-candida agents are quite effective at providing clinical cure for VVC, resistance to the drugs is increasing. For these reasons, the ability of probiotics in maintaining and recovering the normal vaginal microbiota, and their potential ability to resist *Candida* spp. gives rise to the concept of using probiotics for the treatment of vaginal candidiasis³⁵⁻³⁷. Natural defense mechanisms against infections have been described in the vaginal milieu³⁸.

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These include indigenous microbial flora such as lactobacilli, which are believed to interfere with pathogens by various mechanisms. Extending the concept of lactobacilli as endogenous defense mechanisms, both oral and vaginal probiotics have been examined and have shown some efficacy in the context of urogenital health. The actual mechanism of action of lactobacilli in the vagina is probably multifactorial, including immunomodulation in the host, restoration of normal vaginal flora, and interference with pathogen colonization by competing for both nutrients and adhesion sites on the host tissue³⁹. Adherence on the vaginal epithelium is an important virulence factor of *C. albicans*. Co-aggregation of lactobacilli with *Candida* spp. may also be important for the prophylaxis against vaginal infections by preventing the binding to the receptors of the vaginal epithelium. Lactobacilli may block and prevent the *Candida* species' colonization, adhesion, invasion and growth by producing antimicrobial substances like lactic acid, hydrogen peroxide and bacteriocin, which are toxic to *Candida* species^{40,41}. De Alberti et al.⁴² showed that oral consumption of *L. acidophilus* GLA-14 and *L. rhamnosus* HN001, leads to a significant increase in vagina of these species in comparison to baseline and to women in the placebo group. Although it has been suggested that the intestine is a potential reservoir for both vaginal lactobacilli and pathogens (yeasts or bacteria) this study showed not only that consumption of probiotic lactobacilli leads to transient colonization of the vagina but also that the species remained increased, in the vagina, even 1 week after consumption of the probiotics was stopped. These data have been recently confirmed in women with abnormal vaginal microbiota (Nugent score between 4 and 6)⁴³. The use of probiotics in augmenting normal bacterial populations is gradually achieving scientific acceptance. For a few years, probiotics have been used in clinical practice for the treatment of vulvovaginal inflammations and several evaluations have found that are effective against VVC with only few minor side effects⁴⁴, while other studies demonstrated no efficacy in VVC¹⁵. Currently, there is no consensus on the use of probiotics for treating

VVC. Therefore, it is necessary that rigorous randomized, placebo, controlled studies determine the effectiveness and safety of probiotic formulations for the treatment of VVC, and to identify strategic areas for future research.

Lactoferrin is a glycoprotein involved in the host protection against pathogen microorganisms, such as bacteria, viruses and yeasts, and its levels in vaginal secretions increase significantly during infections such as gonorrhea, *Chlamydia*, *cervicitis*, bacterial vaginosis and trichomoniasis⁴⁵ thus highlighting its protective physiological role against pathogens and inflammation. Moreover, both the oral and vaginal administration of lactoferrin has been proven to improve the vaginal microbiota composition by increasing the number of lactobacilli, which contribute to hamper pathogen growth^{46,47}. Evidence shows that it is able to reduce significantly the growth of *Candida albicans* and *C. glabrata*. Interestingly, lactoferrin exerts antibiofilm activity by interfering with *C. albicans* mainly at early stages⁴⁸. Furthermore, Naidu et al.⁴⁹ demonstrated that lactoferrin exerts both adhesion-blocking and detachment properties thus preventing the adhesion of both *C. albicans* and *C. glabrata* to vaginal epithelial cells collected from woman biopsies.

Our study examined the ability of an oral mixture of Lactobacilli with lactoferrin (Respecta[®]) to reduce the recurrence of VVC in a prospective, randomized, double blinded, clinical trial after a conventional therapy with clotrimazole in an induction scheme. Key findings indicated that although the cure rate in the short-term after conventional therapy with topical clotrimazole was comparable in the two groups, a maintenance treatment with probiotics and lactoferrin reduced recurrence rate of candidiasis significantly. In particular, our results showed a significant reduction of recurrence rate corresponding to 33.3% and 29.2% at T4 and T5 respectively, which is somewhat lower than other data reported in literature. The intention to treat women in the luteal phase could become an interesting proposal of maintenance scheme of treatment in patients with RVVC. Finally, the study indicated a

modest, but discernable, late effect on vaginal symptoms that may be related to the potential anti-inflammatory and immunomodulatory effects of investigated lactobacilli and lactoferrin combination. Further studies with larger number of patients and related clinical and immunological outcomes will be mandatory to confirm this suggestive hypothesis.

Acknowledgements

We are grateful to all women that participated to the study. We also thank all the staff of Cebis International, Lugano (Switzerland) for the assistance in data management and clinical operations.

Funding

This clinical trial was funded by Giellepi S.p.A.

Conflict of interest

Rosario Russo is employed by Giellepi; he had no influence on the interpretation of results.

The other Authors have no conflict of interest.

Ethical approval

The clinical study was carried out in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study protocol was approved by the National Committee of Bioethics of Medicines and Medical Devices.

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Tables

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Adult premenopausal women (age 18-50 years).	Vaginal infections different from VVC.
Symptomatic vulvovaginal candidiasis (VVC) and anamnestic history of recurrences.	Women treated with other probiotics or consuming food containing probiotics.
Sexually active women willing to use condom as contraceptive method.	Pregnant or breastfeeding women.
Signed informed consent.	Systemic or local drugs (not indicated in the study protocol).
Not participating in other clinical studies.	Other pathologies (immunodeficiency, diabetes, estrogen-dependent tumors).
Willingness to take the investigational product or placebo.	Hypersensitivity or allergy to antibiotics or any ingredient of investigational product or placebo.
Willingness to collaborate in completing the study procedures.	Use of douching.

Table 2. Demographic characteristics (data are expressed as mean \pm standard deviation) of women participating in the clinical trial.

	Respecta[®] (n=24)	Placebo (n=24)
Age (year \pm SD)	36.5 \pm 7.0	34.3 \pm 7.8
Weight (Kg \pm SD)	64.6 \pm 7.9	62.4 \pm 8.8
Height (cm \pm SD)	166.0 \pm 5.1	165.0 \pm 5.5
BMI (kg/m ²)	23.3	22.7

*BMI = body mass index.

Figure legends

Figure 1. Study flow chart.

Figure 2. Vaginal itching resolution. The graph shows the percentage of women without vaginal itching from baseline (T0) to the end of 6-months follow-up (T5). T1=1 week of therapy; T2= 2 weeks of therapy; T3= 1 month after the end of antifungal therapy; T4= 3 months after the end of antifungal therapy. Black= placebo; Grey=Respecta[®]. **P<0.01 Respecta[®] vs placebo.

Figure 3. Vaginal discharge resolution. The graph show the percentage of women without vaginal discharge from baseline (T0) to the end of 6-months follow-up (T5). T1=1 week of

therapy; T2= 2 weeks therapy; T3= 1 month after the end of antifungal therapy; T4= 3 months after the end of antifungal therapy; T5=6 months after the end of antifungal therapy.

Black= placebo; Grey=Respecta[®]. **P<0.01 Respecta[®] vs placebo.

Figure 4. Overall cure rate. The graph shows the rate (expressed as percentage) of women without any symptoms of VVC. Respecta[®] improved symptoms during the follow-up.

T0=baseline; T1= 1 week of therapy; T2=2 weeks of therapy; T3=1 month after the end of antifungal therapy; T4=3 months after the end of antifungal therapy; T5=6 months after the end of antifungal therapy. Black= placebo; Grey=Respecta[®]. **P<0.01 Respecta[®] vs placebo.

Figure 5. Recurrence rate. The figure shows the rate (expressed as percentage) of women who experienced at least one recurrence of VVC during the follow-up (6 months). Respecta[®]

reduced significantly the recurrences during 6 months. T3=1 month; T4=3 months; T5=6 months. Black= placebo; Grey=Respecta[®]. **P<0.01 Respecta[®] vs placebo.







