

Short Communication

Probiotic and Tea Tree Oil Treatments Improve Therapy of Vaginal Candidiasis: A Preliminary Clinical Study

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Abstract

Recent studies have documented that 29-49% of women has been affected by vulvovaginal candidiasis (VVC) at least once in the lifetime and about 10% of them has history of recurrent VVC.

Tea tree oil (TTO), an essential oil extracted by steam distillation from the leaves of *Melaleuca alternifolia* tree, has been known for many years for its antiseptic properties. Currently, TTO is used in vaginal suppositories (VS) for the treatment of vaginal candidiasis. TTO-VS exhibits an *in vitro* fungicidal activity towards *Candida* spp., only slightly affecting some vaginal lactobacilli population isolated from patients with vaginitis (Di Vito et al. 2015). However, preclinical and clinical studies addressing efficacy and safety profile are still few and not exhaustive, especially with regard to the complex vaginal microbial environment.

On this basis, we have investigated the efficacy and safety profile of a combined therapy with probiotics and TTO-VS on female volunteers with vaginitis in order to find alternatives to fight the growing antimicrobial resistance to the most common synthetic antifungal remedies.

ABBREVIATIONS

TTO: Tea Tree Oil; **VS:** Vaginal Suppositories; **TTO-VS:** Vaginal Suppositories based on Tea Tree Oil; **VVC:** Vulvovaginal Candidiasis; **RVVC:** Recurrent Vulvovaginal Candidiasis

INTRODUCTION

Recent studies have documented a percentage of women comprised between 29% and 49% who have been affected by vulvovaginal candidiasis (VVC), 10% of them with history of recurrent VVC (RVVC) – four or more episodes in a 12-month period [1-3]. In Italy, the prevalence of RVVC is estimated at about 5% out of 16% [4].

Lactobacillus spp. is the dominant bacterium of the vaginal tract in healthy women and an imbalance of the local microbiota can predispose women to infections. Despite the use of efficacious antifungals, there is still a high incidence of recurrence with

associated increase of microbial resistance. Moreover, clinical and epidemiological data suggest that topical treatment for VVC is not always sufficient to eradicate fungal cells from the vaginal microenvironment. Thus, the administration of probiotics incorporating selected *Lactobacillus* strains as an effective strategy for preventing vaginal infections has been suggested [5].

As known, the vaginal biota is mainly formed of *Lactobacillus* spp. – mainly *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii* and *Lactobacillus iners* – producing lactic acid from sugar substrates and making the vaginal microenvironment typically acidic. Furthermore, resident *Lactobacillus* species secrete in cervicovaginal fluid the product of their metabolism, hence creating a barrier against pathogen invasion [6,7]. Recent studies have shown that the presence of *Lactobacillus* spp. in vaginal microbiota is able to counteract the colonization of *Candida* spp. through mechanisms of exclusion, competition and displacement [8].

Other recent studies have evaluated the *in vitro* microbicidal action of some essential oils, such as that of *Melaleuca alternifolia*, against fungal and probiotic strains [9-11].

TTO is an essential oil extracted by steam distillation from the leaves of *Melaleuca alternifolia* (Myrtaceae), native of Australia and well-known for its use in traditional medicine. Currently, TTO is used in some herbal preparations and medicinal products, including VS for the treatment of candidiasis.

However, preclinical and clinical studies are still inconclusive both for efficacy and safety, especially with regard to a complex environment like that of the vaginal microbiota.

The purpose of this short communication is to identify a possible treatment based on probiotics and TTO-VS potentially able to decontaminate the vaginal canal and counteract the fungal colonization by combining the known fungicidal action of TTO with the competitive and immune stimulating one of probiotics. The objective is to lay the groundwork for future clinical study on integrated treatments based on probiotics, administered per os, and TTO-VS in VVC in order to find alternatives to fight the antimicrobial resistance to the most common synthetic antifungal remedies.

MATERIALS AND METHODS

Patients screening and recruiting

Patients with presence of fungal cells in vaginal swabs were recruited from January 2015 to March 2016 in the department of Gynecology at the Sant'Andrea Hospital (UOC San Filippo Neri ASL RME-Rome) and in the department of Altamedica (Rome).

All microbiological tests were performed by the Laboratory of Clinical Microbiology of the San Filippo Neri Hospital (Rome). Inclusion criteria for patients were, besides fungal positivity, the presence of at least one of the symptoms that characterize candidiasis (vaginal discharge, vaginal itching, erythema, dyspareunia, bleeding). Among 147 eligible women, 13 – with different ages and with or without previous vaginal infection – were recruited and treated. The screening was performed using vaginal swabs containing 1 ml of Amies medium, suitable for both microbiological and molecular analysis (ESwab, COPANITALIA, Brescia, Italy). The research of fungi was performed using the standard culture on selective medium and the identification was carried out with MALDI-TOF technology (Vitek MS, Biomerieux, Marcy-l'Étoile, France). The identification was carried out after having isolated single colonies of fungal strains on Sabouraud Dextrose Agar. The treatment received a positive opinion from the Ethical Review Board Committee (Prot. EC 396/16) and all patients gave informed consent. A number of 4 out of 13 women left the study because they did not return for the control, thus 9 completed the treatment.

The treatment included 15 days of oral treatment with 2 cps/day of probiotics made of *Lactobacillus acidophilus* and *S. boulardii* at 2.5 and 4 CFU/cps respectively (Candinorm Capsule® Pegaso Srl Verona - Italy). If the patient was still positive for fungal colonization after the first treatment, the above treatment was followed by further 15 days with VS applied 1/day before bedtime (Candinorm ® VS, Pegaso Srl Verona -

Italy. Composition: 0.5% TTO, *Aloe vera*, GOS, colloidal silica and triglycerides). In addition to vaginal swabs, also vaginal fluids were collected before and after the treatment. Vaginal fluid was obtained from vaginal washing with 1 ml of saline; the recovered lavage fluids were centrifuged at 1000 x g for 10 min at 4 °C to remove cellular debris, then stored at -20 °C until needed.

Voluntary recruitment

In addition to the screening and treatment of patients, 5 female healthy volunteers were recruited to study the kinetics of VS based on TTO (TTO-VS). The healthy female volunteers were treated for 15 days with probiotics and, starting from the 10th day, also with TTO-VS for 5 days (1 TTO-VS/day minimum treatment recommended by manufacturer). During the vaginal treatment, 5 blood samples were collected: the first at time zero (T0) before the application of the first VS, the others after 5 min (T1), 15 min (T2), 3 days (T3), and 5 days (T4). Sera obtained from blood samples were stored at -20 °C until use. Sera were used to study both the presence of TTO major components by GC/MS and the nitric oxide variation by ELISA test.

Nitric oxide, IL-10 and IL-1β expressions

The samples obtained from vaginal washes were examined for the presence of cytokines IL-10 and IL-1β while the plasma samples obtained from female volunteers were studied for the nitric oxide plasma levels. All the analyses were performed using colorimetric ELISA kits (respectively, ab 46034, ab 46052 and ab 65328 from abcam Cambridge Science Park, UK). As said above, vaginal washings were centrifuged at 1000 x g for 10 minutes to remove debris, while serum samples were tested directly without other treatments. The samples were analysed following the manufacturer's instructions and each analysis was in triplicate.

Quantification of *Lactobacillus spp*

Total DNA was extracted by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol "Pretreatment for Gram-positive bacteria". Amplifications were performed with the StepOne Real-Time PCR system (Thermo Fisher Scientific) using the primer pair F Lacto 05 (5'- AGCAGTAGGGAATCTCCA -3') and R Lacto 04 (5'- CGCCACTGGTGTTCYTCATATA -3'), targeting a 375 bp region of the 16S rRNA gene [12]. The 20 µl of amplification mixture contained 10 µl of Powerup® Sybr Green Master Mix (Applied Biosystems), 0.4 µM of each primer and 20 ng of DNA. The amplification conditions consisted of an initial cycle of 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 3 sec and 60 °C for 30 sec. Melting curve analyses were performed by slowly increasing the temperature from 60 °C to 95 °C. Measurements were performed in triplicate and repeated when variation between measurements exceeded 0.5 Ct. Data obtained from amplification were transformed to obtain the number of bacterial cells per ml of transport medium, expressed as colony forming unit (CFU)/ml according to the ribosomal RNA (rRNA) copy number available in the rRNA copy number database [13,14]. Standard curves were made by plotting cycle threshold (CT) values, against dilutions of the quantitative standard for which the number of gene copies was known.

Standard curves were constructed using PCR product of the 16S rRNA gene of *Lactobacillus plantarum* ATCC 14917. The

PCR product was purified with the commercial kit NucleoSpin (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions and photometrically quantified. Different dilutions of purified PCR product (128 ng, 12.8 ng, 1.28 ng, 128 pg, 12.8 pg, 1.28 pg, 128 fg, 12.8 fg, 1.28 pg, 0.128 pg DNA) were used as a template for the standard curve. One nanogram of PCR product corresponded to 2.47×10^9 copies of the fragment gene. The qPCR assays were replicated three times independently.

Gas chromatography coupled with mass spectrometry (GC/MS)

The pure TTO (Lot 140/0000324) and the biological samples were analysed by GC/MSMS following a previously reported method [15]. To check efficiency of TTO compounds extraction from biological samples, we added pure TTO ranging from 0.01% to 1% in sera samples obtained before the treatment with TTO-VS (T0). The calibration curves of two TTO components (Terpinen-4-ol and γ -Terpinene) were carried out to assess the recovery percentage.

All the sera samples obtained from female healthy volunteers were extracted with ethyl acetate in order to assess the presence of TTO-VS compounds.

The value of the GC signal related to the standard Terpinen 4-ol at 3 ppm v/v concentration was measured and compared with the value of the same signal of all the sera samples. We found that the signal values of the sera samples were always lower than the corresponding signal control value of 3 ppm. The solvents and filters were purchased from Sigma-Aldrich (Milan, Italy). The GC/MS analyses were performed on HP GC/MS 6890N-5973N MSD HP ChemStation, equipped with autosampler and HP-5MS column. The following temperature program was applied: 40 °C (4 min), 4 °C/min up to 280 °C (30 min). The mass spectra were measured in the range 35–360 amu. Qualitative analysis was carried out by comparing the retention indices and MS spectra for the obtained peaks with the analogous data from NIST2011 databases.

Statistical analysis

The data obtained in triplicate from each experiment were presented as means \pm standard deviation (SD). The data obtained from PCR assay for cytokines were analysed with t test for paired data, whereas ELISA assay for NO expression was analysed with the variance test for repeated measures. The data were considered significant for values of $P \leq 0.05$.

RESULTS AND DISCUSSION

Patients with positivity to the fungal vaginal search were treated with a mixture of *L.acidophilus* and *S.boulardii*. These probiotics were selected in an attempt to strengthen both vaginal and gut microbiota, usually reservoir of fungal strains [16-18] because a healthy microbiota is able to contrast the fungal colonization and, consequently, the RVVC.

S.boulardii, a yeast of tropical origin classified as probiotic, has generated interest among researchers for its properties in preventing the colonization of fungal strains, altering their adhesion to the substrate [19-21].

Colonization means the presence of a microorganism in or on a host, with growth and multiplication but without any overt clinical expression [22]. In our study, we identified and treated patients that, in routine microbiological analysis, were colonized by fungi showing only some characteristics present in overt fungal infections (Table 1). Furthermore, only 4 out of 9 patients remembered having already suffered from fungal infections prior to recruitment.

After verifying the *in vitro* efficacy of TTO-VS [9] and before proceeding with the administration of VS to patients, we verified on 5 volunteers, during 5 days of treatments, the possibility to detect TTO residues in the bloodstream and/or variations of nitric oxide as mediator of pro-inflammatory response. The results indicate that no components were noticed correlated to the formulation in any of the 5 blood samples taken during the 5 days of treatment (Figure 1) and no statistically significant changes in nitric oxide, a pro-inflammatory mediator, were present during the treatment if compared to time zero (Figure 2). In contrast, 112 TTO components, of which 90% represented by 12 major components (terpinen-4-ol equal to 38% of the total), were detected starting directly from the VS formulation.

Data obtained from the study on patients indicate that, after the first treatment based on probiotics, 55.5% of patients (5/9) were no longer colonized by fungi, and that this percentage reached 77.7% (7/9) when patients, not showing a negativization of the fungal colonization at the first control, received the planned additional treatment characterized by vaginal suppository applications.

Data collected through both questionnaire and medical examination show that all patients at the end of the treatments reported the disappearance of symptoms and a general improvement in well-being. No adverse effects due to treatment were reported.

The ability of *L. acidophilus* to colonize the vaginal canal was evaluated by RT-qPCR analysis performed on vaginal swabs taken before and after treatment. As shown in Table (2), the higher number of lactobacilli found in 7 out of 9 (77.8%) patients analysed after the treatment can be explained by their colonization ability of the vaginal canal. This variation is statistically significant ($P \leq 0.05$) and agrees with data about the *L.acidophilus* ability to colonize the vaginal canal when administered orally [23].

It is well-known that both vaginal microbiota and fungal species are able to influence the immune response by modulating the balance of pro- and anti-inflammatory cytokines, resulting in a physiological immune response also in pregnant women: the IL-10 is expressed 100 times more in healthy women than in those with VB [24-26].

In this study, we investigated the presence of IL-1 β and IL-10 in vaginal washing collected at the beginning and at the end of treatment. Data obtained by ELISA analysis show that all patients turning negative for fungal colonization during the treatment had an increased expression of both IL-10 and IL-1 β in vaginal fluid. While, the two patients with persistence of fungal colonization after the treatments (patients 4 and 10 in Table 2) experienced a decrease in the expression of IL-10 associated with a marked

Table 1: The table shows the physiological data of each patient enrolled and microbiological data obtained from routine tests after each control.

Number	Patient's age	Menopause	Symptoms	Previous fungal infection	Microbial positivity at enrolment	Microbial positivity at first control	Microbial positivity at second control
1	40	NO	VD, VI, E	YES	CP, EC, EF	None	None
2	50	NO	VD, VI, D, B	DNA	CP, EC, EF	None	None
4	43	NO	VD, VI, E, D	YES	CG, EC, EF	CG, EC, EF	CG, EC, EF
5	21	NO	VD, VI, E	NO	CA	None	None
6	46	NO	VI, E, D	NO	CA, EF	None	None
7	52	NO	VI, E, D	DNA	CA, EC, EF	CA, EC, EF	None
9	58	SI	VI, E, D	NO	SC, EC	SC	None
10	34	NO	VD, VI, E, D	YES	CA, EF	CA	CA
14	42	NO	VD, VI, E, D	YES	CA	None	None

Abbreviations: CA: *Candida Albicans*; CG: *Candida Glabrata*; CP: *Candida Parapsilosis*; EC: *Escherichia Coli*; EF: *Enterococcus Faecalis*; SC: *Saccharomyces Cerevisiae*; DNA: Data Not Available; VD: Vaginal Discharge; VI: Vaginal Itching; E: Erythema; D: Dyspareunia; B: Bleeding

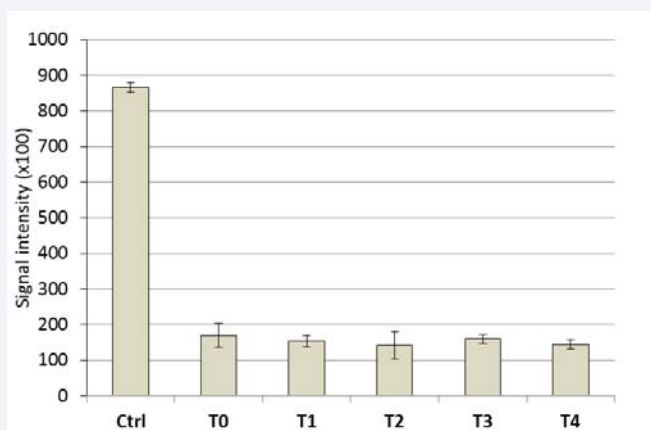


Figure 1 Intensity of terpinen-4-ol GC signal in sera sample of TTO-VS treated volunteers. Each bar represents mean \pm SD of terpinen-4-ol GC signal of the 5 volunteer's sera samples before the treatment (T0) and 5 min (T1), 15 min (T2), 3 days (T3) and 5 days (T4) after TTO-VS administration, respectively. Ctrl represents the 3ppm v/v terpinen-4-ol standard signal intensity value.

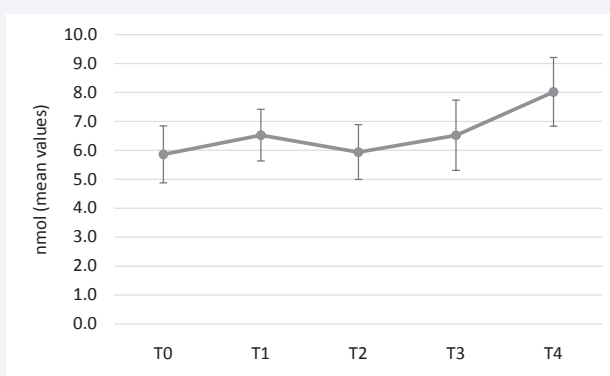


Figure 2 (A) Nitric Oxide expression in blood samples of volunteers. Spots indicate the average levels of NO (nmol) collected prior to the first application (T0) of TTO-VS and after 5 min (T1), 15 min (T2), 3 days (T3) and 5 days (T4). The bars indicate the standard deviation. The variation between samples is not statistically significant ($P > 0.05$).

increase of IL-1 both after the treatment with probiotics and after the cure with TTO-VS. Our data reflect what has been observed in experimental models and pre-clinical literature studies: a right balance of pro-inflammatory and anti-inflammatory cytokines

with preserved vaginal microbiota might be essential for proper immune response in defence against colonization and infection by fungi [24-28].

Table 2: Variation of IL-1 β , IL-10 and *Lactobacillus* spp. The values in the table have been obtained by calculating the variation between the value at recruitment and the one obtained when the microbiological examination became negative or otherwise at the end of all treatments.

Number of Patient	Delta IL-1 β (%) \pm SD	Delta IL-10 (%) \pm SD	Delta <i>Lactobacillus</i> spp (%) \pm SD
1	35 \pm 7.5	29 \pm 3	+3.5 \pm 0.43
2	8 \pm 10.5	18 \pm 2.5	n.a.
4	260 \pm 7	-10 \pm 2.5	n.a.
5	26 \pm 6	4 \pm 2	+0.5 \pm 0.51
6	62 \pm 6.6	52 \pm 2.5	+3.1 \pm 0.60
7	180 \pm 7.5	12 \pm 1.9	+7.8 \pm 0.14
9	234 \pm 7	60 \pm 3	+41.7 \pm 0.27
10	246 \pm 6.5	-5 \pm 2.2	+19.2 \pm 0.72
14	40 \pm 8.1	12 \pm 2	+2.4 \pm 0.11

Abbreviations: IL-1 β : Interleukin-1 β ; IL-10: Interleukin-10; n.a.= DNA amount not available for RT-qPCR analysis.

CONCLUSION

The most common anti-fungal treatments are based on azoles. Even if azoles reduce the frequency of RVVC with no toxicity when applied topically, they are generally not able to eradicate the fungal infection because they are fungistatic. These remedies, although transiently, improve the quality of life of women and require cyclical treatments which gradually generate resistance to the active substances. In contrast, fungicidal treatments are generally resolutive but with important adverse effects especially when administered *per os*.

In recent times, an increasing number of scientific works shows the development of fungal resistance to the most common synthetic remedies. It is known that the development of antimicrobial resistance is an urgent problem that must be overcome both by identifying new molecules with cytotoxic activity and by reducing the widespread use of active molecules. RVVC, in addition to being a problem for about 10% of the female population causing great discomfort, can potentially generate resistors. This requires the use of alternative approaches to common anti-fungal remedies to improve the quality of life of VVC patients and prevent, at the same time, the development of resistances.

The combined treatment with probiotics administered *per os* and TTO-VS applications could be a potentially efficacious remedy able to eradicate the fungal colonization. It appears to be easy to perform, generally well-tolerated unless there is hypersensitivity to the formulation components, and safe.

Compared to conventional therapies, the proposed treatment combines the action of a natural substance, a known fungicide, with the competitive and immune stimulant one of probiotics belonging to the vaginal microbiota. This type of approach is reasonably safe and allows the battle against fungi through two mechanisms. On the one hand, TTO destabilizes the fungal cell by altering the structure of the cell wall [29]; on the other, probiotics, in addition to "undermining" the fungal colonization

(by restoring the vaginal microbiota competing for territory and reducing the fungal adhesion to the substrate), stimulate the physiological defensive response, depressed by the action of the fungus itself. Therefore, the idea is to weaken "the enemy" with a safe and effective natural substance and restore, at the same time, the physiological defence needed to fight the infection. At the same time, this treatment is at low risk for development of resistance since, to date, no fungal strains have developed resistance to TTO. Although the small number of patients studied is a limitation, our work could be an important support for future *in vivo* studies able to identify, on a larger scale, both efficacy and safety in a combined therapy based on probiotics and VS for women with VVC.

In conclusion, our data – although preliminary – are new because for the first time the beneficial activity of the probiotics/tea tree oil combination for an effective vaginal decontamination from fungal organisms has been demonstrated.

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