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In Vitro Activity of Tea Tree Oil Vaginal Suppositories against *Candida* spp. and Probiotic Vaginal Microbiota

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The aim of this work is to evaluate the *in vitro* microbicidal activity of vaginal suppositories (VS) containing tea tree oil (TTO-VS) towards *Candida* spp. and vaginal probiotics. A total of 20 *Candida* spp. strains, taken from patients with vaginitis and from an established type collection, including reference strains, were analysed by using the CLSI microdilution method. To study the action of VS towards the beneficial vaginal microbiota, the sensitivity of *Bifidobacterium animalis* subsp. *lactis* (DSM 10140) and *Lactobacillus* spp. (*Lactobacillus casei R-215* and *Lactobacillus acidophilus R-52)* was tested. Both TTO-VS and TTO showed fungicidal activity against all strains of *Candida* spp. whereas placebo-VS or the Aloe gel used as controls were ineffective. The study of fractional fungicidal concentrations (FFC) showed synergistic interaction with the association between Amphotericin B and TTO (0.25 to 0.08 μg/ml, respectively) against *Candida albicans*. Instead, the probiotics were only affected by TTO concentration ≥ 4% *v/v*, while, at concentrations < 2% *v/v*, they remained viable. TTO-VS exhibits, *in vitro*, a selective fungicidal action, slightly affecting only the *Bifidobacteriun animalis* strain growth belonging to the vaginal microbiota. *In vivo* studies are needed to confirm the efficacy to prevent acute or recurrent vaginal candidiasis. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: Candida albicans; tea tree oil; microbiota; *Lactobacillus* spp.; *Bifidobacterium animalis*; amphotericin B.

INTRODUCTION

Tea tree oil (TTO), an essential oil extracted from leaves of *Melaleuca alternifolia* tree, native to Australia, has been known for many years to the local traditional medicine for its antiseptic properties.

The Australian Aborigines intuitively knew how to use the oil by putting the leaves into a paste to apply on bites, cuts and burns. Their folk wisdom has been passed down for generations, and TTO antimicrobial activity is now being validated through an extensive scientific research (Carson *et al*., 2006; Mondello *et al.*, 2003, 2006). More recently, the European Medicine Agency has recognized its traditional use (Lis-Balchin *et al.*, 2000; Saller *et al*., 1998) and edited a community herbal monograph on *Melaleuca* aetheroleum (EMA, 2013); TTO has been used worldwide for herbalist or pharmaceutical products (SCCP, 2008) including suppositories for the treatment of vaginal candidiasis. However, there is still little evidence that TTO-based preparations could be safe and efficacious for clinical use in a complex microbial environment such as the vaginal one.

Epidemiological and clinical data indicate that a topical treatment for candidiasis is not always sufficient to clear *Candida* spp. from the vaginal micro-environment and that in about 13% of women relapses occur leading to a state of chronic *Candida* infection (Foxman *et al*., 2013). In Italy, the prevalence of these relapses is estimated to be about 5 out of 16 women (Asticcioli *et al*., 2009). The discomfort leads most affected women to use natural products or unqualified medicaments for the treatment and/or prevention of relapses (Cassone, 2015; Watson *et al*., 2012).

Among the interventions based on natural substances, those using TTO, as said, are the most suitable both for low toxicity at concentrations <20% (Hammer *et al*., 2006) and for the fungicidal activity also towards drug-resistant strains. The literature shows that TTO and its major component, terpinen-4-ol (T-4-ol), are active both *in vitro* and *in vivo* with Minimum Inhibitory Concentrations (MICs) ranging from 0.03% to 0.25% and from 1% to 5%, respectively (EMA, 2013). Our previous studies showed the therapeutic potential of TTO and T-4-ol against fluconazole-itraconazole-susceptible or -resistant strains of *Candida albicans* highlighting its fungicidal activity and the ability to accelerate the resolution of the infection in experimentally infected rat vagina (EMA, 2013; Mondello *et al*., 2003, 2006). These previous findings are an important preclinical support for studies of safety and effectiveness of TTO and T-4-ol in human vaginitis.

However, to the best of our knowledge, no study has been developed to test, *in vitro* and *in vivo*, the effectiveness of TTO-based vaginal suppositories (TTO-VS) at the same time on clinical strains of *Candida* spp. and probiotics belonging to vaginal microbiota.

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Based on the above rationale and previous findings, the aim of this work was to evaluate, *in vitro*, fungicidal and bactericidal activities of TTO-based vaginal suppositories (VS) against strains of *Candida* spp. and probiotics belonging to the vaginal microbiota.

In our *in vitro* study, we have used TTO-VS (© Candinorm by PEGASO), regularly sold in pharmacies as a Medical Device Class I, with appropriate studies of toxicity notified to the Ministry of Health and in post-marketing surveillance without any adverse effects notified.

MATERIALS AND METHODS

Microorganisms: For the study of fungicidal action, a total of 17 isolates and three reference strains of *Candida* spp. were used. Fifteen isolates of *C. albicans* were from routine clinical isolates of vaginal swabs in San Filippo Neri Hospital, Rome, Italy. Whereas, fluconazole-itraconazole-susceptible (SA40) or -resistant (AIDS68) strains of *C. albicans,* according to international breakpoint standards, were from the established type collection of the Istituto Superiore di Sanità, Rome, Italy. Reference strains were *C. albicans* ATCC 10231, *C. albicans* ATCC 48274 and *C. tropicalis* ATCC 201380.

The study of probiotic survival was conducted with: three strains of *Bifidobacterium animalis* (BA—from Bologna University, Scardovi Collection of Bifidobacteria), two of which belonging to BA subsp. *lactis* (DSM 10140 and M354) and one to BA subsp. *animalis* (T6); and three strains of *Lactobacillus* spp., two of which obtained from AxiDophilus Pegasus ® (*Lactobacillus casei* R-215, *Lactobacillus acidophilus* R-52) and one reference strain (*Lb. plantarum* ATCC 14917).

TTO, TTO-Products and Probiotics: In this study, TTO-VS (Candinorm®), pl-VS, TTO (*M. alternifolia* essential oil) supplied directly by the manufacturer (Pegaso Srl—Via Pietro Nenni, 5-37024 Arbizzano Negrar—VR) and a reference TTO (*M. alternifolia* Cheel essential oil) (Variati—Milan, Italy) were used (Mondello *et al*., 2003). The TTO supplied (batch 23346) was obtained through steam distillation; the levels of TTO active components (41.7% in T-4-ol and 3.4% in 1,8-cineole) were assessed by gas-chromatography performed by the Statfold Barn Farm (Staffordshire—UK). The composition of the TTO was compliant with the International Organization for Standardization ISO 4730 (range: 30–48% of T-4-ol and 0–15% of 1,8-cineole) (ISO 4730:2004). The TTO-VS are as follows: 0.5% *v/v* TTO, *Aloe vera* gel at a concentration of 1:200 *v/v*, lactic acid, GOS (galacto-oligosaccharides), silica colloidal and triglycerides. The *Lactobacillus* spp. strains used in the study were obtained from a formulation characterized by *Lactococcus lactis* R-1058, *Lactobacillus casei* R-215, *Lb. acidophilus* R-52, R-71 and *Bifidobacterium bifidum* (AxiDophilus® by Pegaso Srl—Via Pietro Nenni, 5-37024 Arbizzano Negrar—VR).

Agar well-diffusion assay: To assess the antimicrobial activity of the TTO-VS, the agar well-diffusion assay was performed. This assay is based on the diffusion of antimicrobial compounds from the formulation through the hydrophilic agar. This method, though not approved by CLSI, had already been used to determine the antimicrobial effects of other Essential Oils (EO) (Singh *et al*., 2007) and to study the antifungal activity of some liquid and semi-solid formulation based on TTO (Thomsen *et al*., 2011). Briefly, wells (with diameter of 5 mm) were punched into each Mueller Hinton (MH) agar plate (Oxoid Hampshire, UK) previously inoculated with 0.5 McFarland solution of ATCC strains (approximately $0.5–2.5 \times 10^3$ cfu/mL in compliance with CLSI guidelines). Wells were then filled with 100 µL of TTO-VS (100% or 50% *v/v* and 25% *v/v* diluted in distilled water with 0.001% *v/v* Tween 80) or control TTO solution (0.5%, 1% and 5% *v/v* in distilled water with 0.001% *v/v* Tween 80), and incubated for 24 h at 37 °C. Tween 80 was included to facilitate oil solubility. After incubation, the diameter of the zone of inhibition was measured to the nearest millimetre. All trials were carried out in duplicate on at least two separate occasions.

Macro-dilution assay for *Candida* spp. strains: The fungicidal action of TTO-VS and TTO was tested by using the macro-dilution assay. A final concentration of 0.001% *v/v* Tween 80 in RPMI 1640 broth (Sigma Chemical Co., St Louis, MO, USA) was included in assays with 0.5% *v/v* of TTO (but not in assays with products). The vaginal suppositories were not diluted with broth but simply dissolved in water bath for not altering the limit effective concentration of TTO (0.5% *v/v*). Before strains inoculation, each TTO-VS was solubilized in a water bath at 37 °C to obtain 2 mL of the final solution in which the strains were inoculated for 24 h. In each experiment a TTO control was included. The TTO control solution was prepared with 0.5% *v/v* TTO in a final volume of 2 mL of distilled water. Product and diluent were vortexed for 10 s before inoculation. Starting from a concentration of 0.5 McFarland, $0.5–2.5 \times 10^3$ cfu of each strain was inoculated in TTO-VS, pl-VS, distilled water and TTO control solution and incubated in a water bath for 24 h at 37 °C. The endpoints could not be visually determined, and the minimal fungicidal concentrations (MFCs) were therefore determined by spreading the 100 µL of solution onto MH agar and chromagar (Oxoid, Hampshire, UK) and incubated again for 24 h at 37 °C. The MFC was determined as the lowest concentration resulting in no growth of subculture following incubation. Tests were carried out in triplicate on at least three separate days.

Macro-dilution assays for probiotic strains: Were performed to evaluate the probiotics' vitality in the presence of TTO-VS. The growth of each strain was studied in the presence of TTO-VS, pl-VS (both performed as described in macro-dilution assay for *Candida* spp. strains) and physiological solution; *Lb. acidophilus* R-52*, Lb. casei* R-215 and *B. animalis* subsp. *lactis* (DSM 10140) strains were inoculated at 5×10^{10} for *Lactobacillus* spp. and 4×10^{10} cfu/mL for *B. animalis* subsp. *lactis.*, respectively. The samples were incubated at 37 °C for 24 h; the survival rate for different strains was determined by plating 10 µL, 100 µL and 1 mL from each sample onto agar medium (deMan-Rogosa-Sharpe (MRS) for lactobacilli, and Tryptone-Phytone-Yeast extract (TPY) for bifidobacteria, both from Merck, Darmstad, Germany), and incubated for 4 days at 37 °C in anaerobiosis (Biavati and Mattarelli, 2012). The survival rate was calculated by comparing cfu/mL at initial and final time of sampling. A bactericidal effect was considered

as a 3 log decrease in the cfu/mL. Tests were carried out in duplicate on at least two separate days.

Broth micro-dilution assay: *Lactobacillus* spp. and BA strains were cultivated in MRS and TPY agar medium, respectively (Biavati and Mattarelli, 2012). They were incubated at 37 °C for 24 h in anaerobiosis (Anaericult Merck, Darmstad, Germany). The broth micro-dilution test was performed in compliance with CLSI (CSLI, 2006) with the following modifications: (i) MRS and TPY broths were utilized for *Lactobacillus* spp. and BA, respectively; (ii) Tween 80 was utilized as emulsifier at 0.001% *v/v*. The strain inoculum was obtained by starting from 0.08 to 0.130 optical density (OD = 600 nm, approximately 5.0×10^5 cfu/mL) of bacterial suspension in physiological solution. The final concentration of TTO ranged from 0.0078 to 4% *v/v*. After 24 and 48 h of incubation at 37 °C in anaerobiosis, bacterial growth was evaluated by optical density at 600 nm by using a spectrophotometer (Multiskan EX, Thermo Scientific). The MIC was defined as the lowest concentration in absence of both visible growth and the OD data.

Time killing and viability assays: TTO was tested against the fluconazole-itraconazole-resistant *C. albicans* strain as reported elsewhere (Mondello *et al.*, 2003). Briefly the fungal inoculum was prepared by growing azole-resistant strain isolated on Sabouraud dextrose agar SDA for 48 h at 35 °C. The inoculum suspension was prepared by picking five colonies with a diameter of at least 1 mm and suspending them in sterile distilled water. The cell density of the suspension was adjusted to 2.5×10^6 cells per mL. A further dilution was made by inoculating 200 µL of the cell suspension in glass tubes containing 1.8 mL of RPMI 1640 broth solution (with 0.001% *v/v* Tween 80 and 1% *v/v* TTO at pH 7), and in other tubes containing 1.8-mL Sabouraud broth solution (with 0.001% *v/v* Tween-80 and 1% *v/v* TTO at pH 5). The final cell density was approximately 2.5×10^5 cfu/mL. Glass tubes containing only RPMI with Tween 80 pH 7 and Sabouraud broth with Tween 80 pH 5 were the negative control. All tubes were incubated by shaking them at 35 °C to ensure uniform essential oil dispersion; the incubation was stopped with cold acetate buffer (pH 3.5) after 2, 5, 10, 15, 30 and 60 min, and 100-µL aliquots were removed for cfu enumeration. The cfu were determined by making appropriate dilutions in acetate buffer, plating 100 µL of each dilution on SDA with chloramphenicol (50 mg/L, Sigma) and incubating for 48 h at 35 °C. In control experiments, acetate buffer alone had no inhibitory effect on *in vitro* growth of *C. albicans*. Each experiment was performed in triplicate, independently. A fungicidal effect was defined as a 3 log decrease in the cfu/mL, or a >99.9% killing over a specified time. In viability assays the fungal inoculum was prepared as in time kill studies, but the final fungal cell concentration ranged from 1×10^6 to 3×10^6 cfu/mL.

Checkerboard titration method: 96-well microplates were used, each one containing Tryptic Soy Broth (TSB) with concentrations ranging from 1/8 to 8 × MIC for essential oils (1 × MIC = 0.5%), from 1/8 to 16 × MIC for Amphotericin B (1 × MIC = 0.5 µg/mL) and combined with each other on the plate in a checkerboard style. The fungal inoculum (*C. albicans* ATCC 48274) was 1.0×10^5 cfu/well. The microplates were incubated for 24 h at 37 °C. The microplates were incubated for 24 h at 37 °C. The Fractional Inhibitory Concentration (FIC) Index and the Fractional Fungicidal Concentration (FFC) Index were calculated in compliance with international guidelines (EUCAST, 2000). Synergism was defined as FIC or FFC index < 0.5; additivity FIC or FFC index between 0.5 and 1; indifference FIC or FFC index between 1 and 2 and antagonism FIC or FFC index > 2 (EUCAST, 2000). Each experiment was performed in quadruplicate, independently.

Statistical analysis: The data obtained in triplicate from each experiment were presented as means ± stanndard deviation (SD) in both isobologram or istogram for continuous variable and bar graph for ordinal variables. The data obtained from time killing assay were analysed with Student's test in order to identify the significance intra and intergroup. The data were considered significant for values of $P \leq 0.05$. The data obtained from probiotics study were submitted to the analysis of variance (ANOVA) by means of the Co Stat 6.3 software (CoHort Software, Monterey, CA, USA) to determine the significance of ANOVA sources (bacterial strain, TTO level and their interaction), according to a completely randomized factorial scheme at three replicates. The Student–Newman–Keuls (SNK) test at $P < 0.05$ was adopted to separate the levels of significant ANOVA sources.

RESULTS AND DISCUSSION

The broth macro-dilution assay was performed to study the fungicidal activity of TTO-VS and TTO over a 60-min interval. All *Candida* strains tested, including the reference strains and the azole-resistant strain AIDS 68, were shown to have no growth in either TTO-VS or TTO, with a fungal load reduction of around 1.5 log. The data are in substantial agreement with those obtained by the viability test that showed 100% of fungicidal activity starting from 24 h of TTO treatment (Mertas *et al.*, 2015; Mondello *et al.*, 2003). A fungicidal activity, though to a lower magnitude, was also shown against the azole-resistant *C. albicans* AIDS68 strain, as shown by >80% drop of cfu/mL within 60 min at a TTO concentration of 0.5% *v/v* (Fig. 1a). Importantly, our experiments also showed that the pH 5 does not significantly alter the fungicidal activity of TTO ($P = 0.15$), supporting the notion that VS-TTO activity in the vagina would not be counteracted by elevation of the vaginal pH during *Candida* infection (Fig. 1a–b) (Mondello *et al.*, 2003; Danby *et al.*, 2012; Cassone, 2015).

Moreover, we wondered about what could be the highest concentration applicable *in vivo* and able to give the best results with the least damage. Concentration of 1% [corresponding to the minimal bactericidal concentration (MBC) of *B. animalis*] seems to be a good candidate because it very slightly affects the beneficial microbiota and achieves the best fungicidal activity in a reasonably short time as already shown by our time killing (Fig. 1b).

Evidence supports what has been verified among preclinical models of vaginitis in female rats (Mondello *et al.*, 2003, 2006) and suggests future applications that are safer than the one reported in the only clinical trial within EMA documents, where 28 women were treated with vaginal capsules of 2 g with TTO at 10% (Belaiche, 1985). In our opinion, a 10% concentration would be

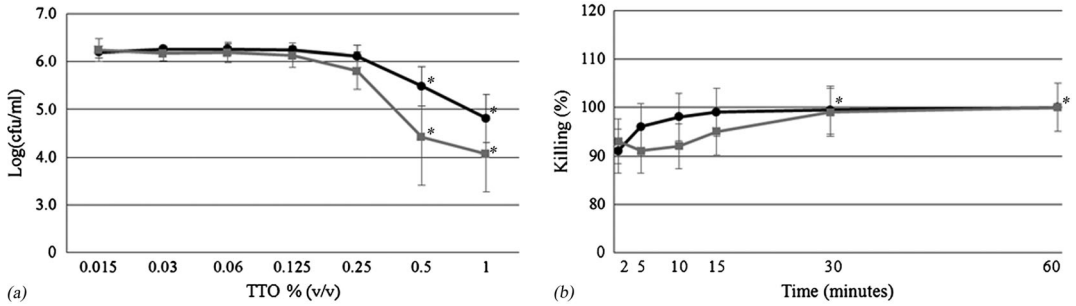

Figure 1. Fungicidal activity and killing of TTO on azole-resistant *C. albicans* strain. (a) Fungicidal activity of TTO at different concentrations against azole-resistant strain (≈1–3 × 10^6 cfu/mL) at pH7 (black filled circles) and pH 5 (grey filled squares), at 60 min. (b) Killing of azole-resistant strain (≈2.5 × 10^5 cfu/mL) by 1% *v/v* TTO at pH 7 and pH 5. Data are expressed as mean ± SD. The data highlighted with * are statistically significant ($P < 0.05$).

able to eradicate the infection while destroying the vaginal microbiota.

We also tested the TTO-VS fungicidal activity by the agar well-diffusion assay to evaluate, in the presence of a solid formulation, the efficiency of a method already employed, with some uncertainty, with liquid and semi-solid TTO-formulations (Thomsen *et al*., 2011). Our data show that also the solid formulation offers unreliable results because the inhibition zones around the TTO-VS wells are not well visible. Therefore, the agar well-diffusion test is not suitable for our purpose, perhaps due both to the solid nature of the formulation and to the low concentration of the TTO diffusing inside the hydrophilic agar.

In addition, we wanted to study the presence of possible synergy between the TTO and Amphotericin B, one of the most powerful and toxic fungicides, to evaluate the opportunity to make formulations for topical use with a lower concentration of this synthetic component by addition of low concentration of TTO. The above, in order to obtain a reduction of the toxicity of topical formulation.

To date, some studies have analysed the interaction of the TTO with both Amphotericin B (Rosato *et al*., 2008; Van Vuuren *et al*., 2009) and with other antimicrobial substances (Forrer *et al*., 2013; Yap *et al*., 2013; Low *et al*., 2011). In literature, the data of synergy require further studies because it was conducted with different methods, quality of EO and interpretations of the data (Odds, 2003; EUCAST, 2000). Our results comply with the studies that identify a dose-dependent synergism between TTO and Amphotericin B (Rosato *et al*., 2008; Van Vuuren *et al*., 2009).

The analyses of the combination of TTO and Amphotericin B were performed in triplicate and the fractional fungicidal concentrations (FFCs) were calculated as the MFC of the combination of the TTO and Amphotericin B divided by the MFC of TTO or Amphotericin B alone. Our data showed synergistic effects at concentrations of 0.25 μg/mL for Amphotericin B and of 0.054 μg/mL for TTO (1 μg/mL of TTO corresponding to ≅0.11% *v/v*). The synergistic effect on *C. albicans* ATCC 48274 is graphically shown by isobologram constructed on the basis of the checkerboard experiment (Fig. 2).

The importance of a suitable vaginal microbiota to improve and resolve the physiological defence against infection is well known. Hence, we investigated the impact of the VS-TTO on probiotic strains belonging to the vaginal microbiota.

As described in Materials and methods, six strains of probiotics where tested with different TTO concentrations, and three strains of the former were tested in

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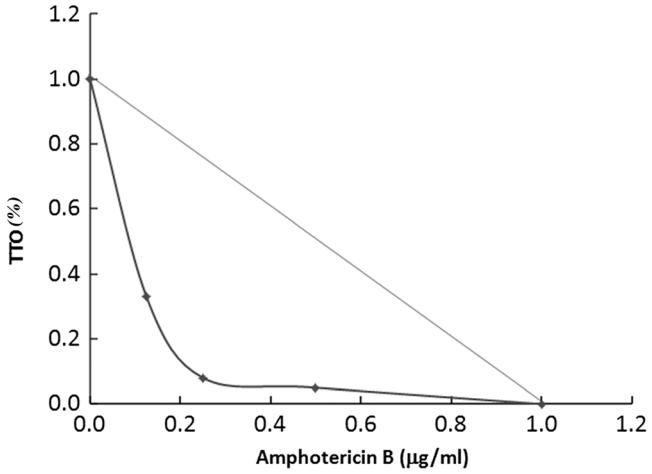

Figure 2. Isobologram describing the synergistic effect of TTO with Amphotericin B against *Candida albicans* ATCC 48274. The points plotted on the isobologram were based on the results of checkerboard assay.

the presence of TTO-VS. Particularly, two strains of *Lactobacillus* spp. (*Lb. casei R-215* and *Lb. acidophilus R-52*) were analysed as probiotics used in pharmaceutical formulations able to colonize the vaginal micro-environment. Moreover, the strain of BA subsp. *lactis* DSM 10140 was analysed as it is the most resistant species to environmental stress and the most utilized species as probiotics in food and pharmaceutical preparations (Amund *et al*., 2014; D'Aimmo *et al*., 2007). The data obtained by the broth macro-dilution test showed that the TTO-VS had no inhibitory action on *Lactobacillus* spp growth, while there was a strong growth inhibition on BA (colony counts: $20 \times 10^4 \pm 0.1 \times 10^4$ cfu/mL). In both cases, no significant change in growth was shown following bacteria inoculation in pl-VS compared to the control incubated only in saline, which showed confluent growth. These results were confirmed in all of the six strains of probiotics, as shown by MIC values in Table 1, when tested in presence of serial dilutions of TTO using broth microdilution tests. As shown in Fig. 3, the interaction between TTO and *Lactobacillus* sp. and BA subsp. *lactis* exhibits a remarkably different behaviour between the two strains: at the highest TTO level (4% *v/v*), the two strains were strongly inhibited to a similar extent; at the seven intermediate

Table 1. *In vitro* antimicrobial activity of TTO and TTO-VS *vs* probiotics and Candida spp. strains. MIC = minimal inhibitory concentration, MBC = minimal bactericidal concentration, MFC = minimal fungicidal concentration, (*) = MFC_{90}, the column of TTO-VS show weak (+), strong (+++) or no (−) growth, n.d. = not determined

Species	Strains	MIC TTO (%*v/v*)	MBC/MFC TTO (%*v/v*)	TTO-VS
Bifidobacterium animalis subsp. *lactis*	DSMZ 10140	0.5	1	+
Bifidobacterium animalis subsp. *lactis*	M 354	0.5	1	+
Bifidobacterium animalis subsp. *animalis*	T6	0.5	1	+
Lactobacillus acidophilus	R-52	4	4	+ + +
Lactobacillus casei	R-215	4	4	+ + +
Lactobacillus plantarum	ATCC 14917	2	4	+ + +
Candida albicans	ATCC 48274	0.25	0.5	−
Candida albicans	ATCC10231	0.25	0.5	−
Candida tropicalis	ATCC201380	n.d.	0.25	−
Candida albicans	AIDS68	0.25	0.5	−
Candida albicans	SA-40	0.25	0.5	−
Candida albicans	14 human isolates	n.d.	0.25(*)	−

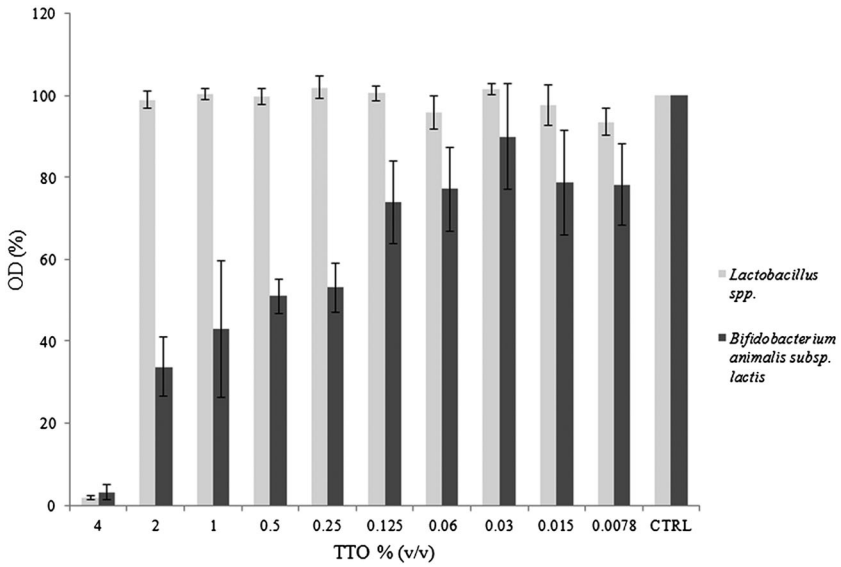

Figure 3. Antimicrobial activity of TTO against *Lactobacillus* spp. and BA by the broth micro-dilution method. TTO was used at concentrations ranging from 0.0078 to 4% *v/v*. CTRL, negative control. Data are expressed as mean ± SD. All data are shown to be highly significant ($P < 0.001$; see discussion).

levels (from 0.0078 to 2% *v/v*), *Lactobacillus* spp. was not affected, whereas BA subsp. *lactis* showed a decreased growth roughly proportional to TTO level. More specifically, a highly significant inverse correlation ($r = -0.94^{**}$) was found between TTO and relative OD in BA subsp. *lactis*, indicating an 8% decrease in OD per each 1% increase in TTO (equation not shown). Owing to non-homogeneous variances (significant Bartlett test), relative OD data were transformed into the respective arcsin*square root values, prior to ANOVA. All ANOVA sources (strain, TTO and their interaction) were shown to be highly significant ($P < 0.001$).

As already observed in the case of bacterial vaginosis (Hammer *et al.*, 1999). The probiotics' MICs are higher than those identified for strains of *C. albicans*. This greater resistance of *Lactobacillus* spp. is an interesting result if it is considered that the vaginal microbiota is predominantly characterized by acidophilic *Lactobacillus* spp. Our data hypothesize the possibility of TTO exerting a selective action against pathogenic strains of *Candida* species without significant alterations of the beneficial vaginal microbiota.

CONCLUSION

Our data support both the traditional use in the gynaecological field and the notion that TTO vaginal suppositories, easy to use and fungicidal against the pathogen only, could be combined with the local standard therapy, generally fungistatic, for the *Candida* spp. vaginal infection. This in order to reduce relapses and avoid the chronicity of the disease, especially in cases of azole-resistant pathogens.

It is known that synthetic and natural products could be toxic. For EOs, particularly tea tree oil, toxicity was generally demonstrated at high doses (Hammer *et al.*, 2006). Our findings support the notion that combined formulations of both synthetic and natural antifungal substances could be active and non-toxic because of the reduction of doses of both synthetic and natural substances used for topical applications.

In summary, our study underlines the need for considering appropriate investigations in adequate controlled and randomized clinical trials to further support a

possible use of TTO-based suppositories to fight chronic vaginal infections by *Candida* spp.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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