Effectiveness of the Association of 2 Probiotic Strains Formulated in a Slow Release Vaginal Product, in Women Affected by Vulvovaginal Candidiasis

A Pilot Study

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Background: Vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis after bacterial vaginosis, and it is diagnosed in up to 40% of women with vaginal complaints in the primary care setting. Among *Candida* spp., *Candida albicans* is the most common infectious agent. The treatment of choice for uncomplicated VVC is achieved with single-dose or short-course therapy in over 90% of cases. Several topical and oral drugs are available, without evidence for superiority of any agent or route of administration. In any case, most classic treatments are unable to significantly offer a protection against possible recurrences. In recent years, probiotics are emerging as a new strategy to counteract VVC. In fact, they are well known for their ability to lower intravaginal pH, thus establishing a barrier effect against many types of yeasts. Some strains are also able to exert additional and more focused antagonistic activities mediated by specific molecules such as hydrogen peroxide and bacteriocins. For example, *Lactobacillus fermentum* LF5 (CNCM I-789) was successfully tested in 4 human trials involving a total of 340 women reporting VVC at enrollment. In any case, the way used to deliver probiotics to the vaginal environment represents a crucial point. The aim of this work was to first select 1 or more probiotic strains in vitro with an antagonistic activity on *Candida* yeasts and then to perform an in vivo human pilot study using an association of the most promising and active bacteria.

Methods: For this purpose, 2 probiotic strains Probiotical S.p.A (Italy) were selected based on their strong in vitro inhibition activity toward 4 particular *Candida* species, namely *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* and subsequently tested in a human intervention pilot trial involving 30 women with VVC. The probiotics used, *L. fermentum* LF10 (DSM 19187) and *Lactobacillus acidophilus* LA02 (DSM 21717), were administered by means of slow release effervescent vaginal tablets (ActiCand 30 product). The main endpoint was the assessment of the establishment and maintenance of a barrier effect against *Candida* yeasts in women suffering from VVC. Thirty female subjects who were diagnosed with VVC by both microscopic examination and yeast culture were enrolled in the study and directed to apply a vaginal tablet once a day for 7 consecutive nights, followed by 1 tablet every 3 nights for a further 3-week application (acute phase) and, finally, 1 tablet per week to maintain a long-term vaginal colonization against possible recurrences. A medical examination of each patient was performed at enrollment (d_0), at the end of the first 4 weeks of treatment (d_{28}), and at the end of the second month of relapse prevention (d_{56}). The visual and microscopic examination was always accompanied by microbiological analyses of vaginal swabs to assess the presence of *Candida*. A statistical comparison was made between d_{28}, or d_{56}, and d_0, and between d_{56} and d_{28} to quantify the efficacy against possible recurrences.

Results: The administration of the product ActiCand 30 was able to significantly solve *Candida* yeast symptoms after 28 days in 26 patients out of 30 (corresponding to 86.6%, $P < 0.001$). At the end of the second month, recurrences were recorded, albeit not particularly serious, in only 3 out of 26 patients (11.5%, $P = 0.083$) who were found to have fully healed at the end of the first month of treatment. This is a further confirmation of the long-term barrier effect exerted by the product.

Conclusions: VVC has a very high incidence as 70% to 75% of women report at least 1 episode during the life. Many treatments are currently available but, despite a relatively high effectiveness in the relief of symptoms typically associated with acute infections, they are generally unable to offer a long-term protective barrier against possible recurrences. This study demonstrated the ability of ActiCand 30 to not only solve *Candida* infections in a very high percentage of women, but also to exert a long-term physiological defense due to the colonization of vaginal microbiota and adhesion of the mucosa to the epithelial cells. The special formulation of ActiCand 30, consisting of slow release effervescent vaginal tablets, is able to mediate 2 types of barrier effects, the first represented by the formation of an anaerobic environment due to the release of CO_2 and the second guaranteed by the colonization and adhesion to the vaginal epithelium of the 2 probiotics *L. fermentum* LF10 and *L. acidophilus* LA02.

Key Words: vulvovaginal candidiasis (VVC), vaginal microbiota, probiotic strain, barrier effect

(*J Clin Gastroenterol* 2012;46:S73–S80)

The composition of the urogenital microflora is crucial for the health and well-being of women.[1]

In the vaginal environment, many different groups of microorganisms, either commensals, opportunistic pathogens or probiotics, coexist in equilibrium with each other and with the guest. They are subject to qualitative and quantitative changes over the years and in response to both exogenous and endogenous factors. As the behavioral exposures may challenge the perpetuation of the *Lactobacillus* population, the intrinsic stability of the resident microflora is paramount to women's health.[2]

Several factors can cause an imbalance in the vaginal microflora which, in turn, can lead to the onset of vaginitis or chronic forms of vaginosis. Very common are those resulting from unrestricted growth of yeasts belonging to

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the *Candida* genus, of which the most well-known species is represented by *Candida albicans*. This dimorphic yeast is a commensal that colonizes skin, the gastrointestinal, and the reproductive tracts.[3,4]

It is the main causative agent of mycotic vulvovaginitis, although in recent years infections caused by different species such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* have steadily increased.[5,6]

The pathogenesis and prognosis of candidial infections are affected by the host immune status and also differ greatly according to disease presentations.

The incidence is very high, as 70% to 75% of women have at least 1 case of vulvovaginal candidiasis (VVC) during their life.[7,8] Equally important is the fact that 40% to 50% of subjects have 1 or more recurrences after the apparent resolution of the first infective episode.[9] Moreover, 25% to 40% of women who are culture positive for the *Candida* species in the vaginal area are asymptomatic carriers.[10]

VVC is regarded as an opportunistic infection of endogenous origin; therefore, it is not a sexually transmitted disease.[11] After bacterial vaginosis, VVC is the second in frequency among vaginitis, which are the leading cause of gynecologic examination in western countries.[12]

The diagnosis should be made on the basis of clinical observations reported by the gynecologist and, if doubts remain, it must be confirmed by a microscopic assessment of fresh tissue and the execution of a microbial culture before starting a specific pharmacological therapy.[13]

Some studies have identified some predisposing conditions that often, precisely by altering the balance of vaginal ecosystem, enable its development, the appearance of typical symptoms of the infection, and determine recurrences.

Contributing factors could be the use of antibiotics, cortisone, genetic and racial factors, diseases of the immune system, stress, diabetes, increased local temperature and moisture (wet swimsuits, wet linen at the gym), excessive use of douches and detergents, or a diet rich in glycogen and hormonal contraceptives.[14–18] Recurrences are more frequent in perimenstrual period.

Menopause, ageing, and pregnancy, due to the hormonal changes involved, are conditions able to encourage *Candida* growth. In fact, many women have their first episode or a relapse just during these periods.[19]

Candida causes burning, itching, and discomfort and, in case of recurrences, often has a major impact on the quality of social, psychological, and sexual life of the woman.

Management approaches include treating each individual episode or using prophylactic antimycotic measures.[20] Previously, several prophylactic regimens were advocated that involved the use of intravaginal antimycotic agents either daily or weekly or oral ketoconazole for approximately 6 months.[21,22] Although these measures are generally regarded as effective in the treatment of acute infections, they are frequently unable to offer a significant protection against possible recurrences of VVC. Furthermore, they are inconvenient and expensive, and oral ketoconazole was associated with an unacceptable risk of hepatotoxic effects.[23]

In relapses, and as prophylaxis and prevention, the use of specific probiotic microorganisms is consolidating with the aim of restoring the physiological balance of vaginal ecosystem, mainly attributed to the presence of lactobacilli and the so-called Döderlein complex.[24] The use of live microorganisms with a probiotic value has, in fact, been indicated for some years by many gynecologists as an alternative or complementary therapy to the use of traditional topical medicines with antibacterial or antimycotic activity.[25] The oral and vaginal administration of probiotics is taken into account and considered useful also during pregnancy.[26]

Probiotics are well known for their ability to lower the intravaginal pH, thus establishing a barrier effect against many types of yeasts.[27] Some strains are also able to exert additional and more focused antagonistic activities mediated by specific molecules such as hydrogen peroxide and bacteriocins.[28] For example, *Lactobacillus fermentum* LF5 (CNCM I-789) was successfully tested in 4 human trials involving a total of 340 women reporting VVC at enrollment (data not published).

In any case, the dosage form and strategy adopted to deliver probiotics to the vaginal environment represents a crucial point. Formulations based on oily probiotic suspensions are most often unable to guarantee the greatest efficacy of the beneficial bacteria used. For this reason, alternative forms have been recently investigated, specifically hydrogels, capsules, and tablets.[29]

In light of the above, an association of probiotic strains was selected in vitro on the basis of their ability to directly antagonize different *Candida* species. As a second step, a combination of the 2 specific probiotic strains *L. fermentum* LF10 (DSM 19187) and *Lactobacillus acidophilus* LA02 (DSM 21717), administered in the form of a new commercial product, ActiCand 30, was investigated in vivo for its ability to create and maintain a vaginal microenvironment that did not encourage the establishment, propagation, or persistence of a *Candida* infection.

MATERIALS AND METHODS

In Vitro Assessment of the Antagonistic Activity of Selected Probiotic Strains Against *Candida* Yeasts

The in vitro inhibitory activity of 3 probiotic strains, namely *L. fermentum* LF10 (DSM 19187), *L. fermentum* LF11 (DSM 19188), and *L. acidophilus* LA02 (DSM 21717), against 5 yeasts belonging to 4 different *Candida* species was quantified at the Biolab Research (Novara, Italy).

The probiotic strains used were characterized at the species level by species-specific polymerase chain reaction using the primers Lfpr/Ferm II for *L. fermentum*,[30] and ACI/16S II for *L. acidophilus*.[31] With regard to the individual biotypes, the molecular fingerprinting was obtained using a pulsed-field gel electrophoresis analysis for all strains.[32]

More specifically, *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. albicans* ATCC 90028, and *C. glabrata* ATCC 2001 were used. The inhibitory results of fresh broth cultures of individual probiotic strains grown overnight in De Man, Rogosa, and Sharpe (MRS) broth (Merck Chemicals, Milan, Italy) were compared with freeze-dried cultures of the same bacteria. In particular, fresh broth cultures were used with all *Candida* strains, whereas freeze-dried samples were tested against *C. albicans* ATCC 10231, chosen as an indicator of activity. Before being used in the experiment, individual *Candida* strains were grown in Sabouraud Dextrose Broth (BD Italia, Milan, Italy), an optimal medium for yeasts, for 48 hours in aerobic conditions. Each probiotic was then cocultured in the same broth (MRS) inoculated with one of

the above-mentioned *Candida* microorganism. The ratio between the inocula of probiotic and yeast was 1:3 in favor of the latter. Incubation was performed in aerobiosis at 37°C for 48 hours. Each culture (1 mL) was sampled after 24 and 48 hours of incubation for the selective enumeration of the yeasts, performed on Yeast extract glucose chloramphenicol (YGC) agar medium (Sigma-Aldrich, Milan, Italy). YGC agar plates were incubated in aerobic conditions at 25°C for 24 to 48 hours to allow the growth of any yeast present. Colonies were then counted and results expressed as number of colony-forming units (CFU)/mL.

In Vivo Study Design

Thirty female patients (age between 23 and 64 y, mean 40.9 ± 11.1, menopausal women 8) were enrolled in the study between May and June 2011 at the Casa di Cura "San Pio X" Private Clinic (Milan, Italy). Informed written consent was obtained from all participants involved in the study.

Eligible subjects were at least 18 years old, had active, acute vulvovaginal candidiasis (total severity score = 3), had a positive result from microscopic examination of vaginal secretions with 10% potassium hydroxide; and had at least 4 documented episodes of vulvovaginal candidiasis in the last 12 months.

The severity score was based on the presence of symptoms (eg, itching, irritation, and burning) and vulvovaginal signs (eg, erythema, edema, and excoriation, or fissures). The severity of each sign or symptom was scored on a scale of 0 (absent or normal) to 3 (severe).[33] The level of vulvovaginal discharge was not scored.

Patients were excluded from the study if the microscopic findings could not be confirmed by culture. Other exclusion criteria were pregnancy, mixed infections, known seropositivity for the human immunodeficiency virus, and treatment with antifungal agents or other probiotic-containing products, even if taken orally, in the previous 2 months.

After enrollment in the study, all patients received the product ActiCand 30 (Probiotical, Novara, Italy), formulated in slow release, slightly effervescent vaginal tablets containing at least 0.4 billion live cells of each probiotic *L. fermentum* LF10 and *L. acidophilus* LA02. In addition, each tablet contained 273 mg of arabinogalactan and 332 mg of fructo-oligosaccharides, 2 prebiotic fibers used to enhance vaginal colonization by the 2 probiotics. The slight effervescence was mediated by the presence of 64 mg of citric acid and 56 mg of sodium bicarbonate and was particularly intended to rapidly create an anaerobic vaginal microenvironment able to favor the growth of probiotics and, on the other side, to exert a barrier effect against *Candida* yeasts that, for the fact of being respirer microorganisms, are unable to grow in anaerobic conditions.[34]

The protocol adopted was mostly in accordance with the instructions for use of the commercial product; therefore, patients were directed to apply a vaginal tablet once a day for 7 consecutive nights, preferably before going to bed. Then, to maintain the vaginal colonization by the 2 probiotics, patients used 1 tablet every 3 nights for a further 3-week application. The acute treatment lasted, therefore, for 4 weeks in total. In the following month, to maintain a long-term vaginal colonization against possible recurrences, patients used 1 tablet per week.

Patients were prohibited from using topical vaginal or other systemic antifungal agents at any time during the study. They were also prohibited from using antibiotics or other probiotic products, even if taken orally, throughout the duration of the study.

A vaginal medical examination of each patient, which included obtaining vaginal swabs for yeast culture, was directly performed at the Gynaecology Unit of the Private Clinic at baseline, at the end of the first 4 weeks of treatment and at the end of the second month of relapses prevention.

Collection of Vaginal Swabs and Yeast Cultures

At each visit (baseline, d_0; end of the acute treatment, d_{28}; end of the second month, d_{56}), vaginal swabs were obtained for culture. Once a swab sample was collected, it was placed immediately into a polypropylene screw-cap ESwab transport tube filled with 1 mL of Liquid Amies transport medium (BD Italia), stored at 4°C and delivered to the laboratory within 24 hours after collection. On receipt of clinical isolates, the ESwab specimen tubes were briefly vortexed, and then the swab was removed according to the manufacturer's instructions. Swab medium (100 μL) was inoculated onto Yeast extract dextrose chloramphenicol (YGC) agar (Sigma-Aldrich). All the agar plates were incubated in aerobic conditions at 25°C for 24 to 48 hours to allow the growth of any yeast present.

A subsequent identification at the species level was performed with the use of the API 20C AUX system (BioMerieux, Florence, Italy). More specifically, 2 yeast colonies were picked from each agar plate, individually inoculated in molten (50°C) API basal medium ampoules and each suspension was standardized to a density below 1 + (lines can be clearly distinguished) on a Wickerham card. Each cupule of the strip was inoculated (20 cupules; approximately 0.2 mL each) by using a Pasteur pipette, and the trays were incubated for 72 hours at 30°C. Cupules showing turbidity significantly higher than that of the negative control cupule (cupule "0") were considered positive. Identification was made by generating a microcode and using the API 20C AUX Analytical Profile Index.[35] 2 colonies were used to be more confident of the result.

The assessment of the presence of yeasts in vaginal swabs, and *Candida* identification at the species level, were performed at the Biolab Research.

Statistical Analysis

A subject positive for *Candida* infection, as confirmed by both microscopic examination and yeast culture, was marked with "X". Paired *t* test statistical analysis was used to weigh the results and compare them between d_{28} and d_0, d_{56} and d_0, and d_{56} and d_{28}. Differences were considered significant at $P \leq 0.05$.

RESULTS

In Vitro Antagonistic Activity of Selected Probiotic Strains Toward *Candida* Yeasts

The molecular fingerprinting of the 3 strains investigated for their in vitro activity against *Candida* is reported in Figure 1.

All the probiotics demonstrated considerable antagonistic activity against the 5 *Candida* strains tested, with the 2 *L. fermentum* significantly more effective than *L. acidophilus* LA02. No significant differences were recorded between fresh broth cultures and freeze-dried samples of each probiotic bacterium, thus demonstrating that, despite a reasonable moderately longer lag phase at the very

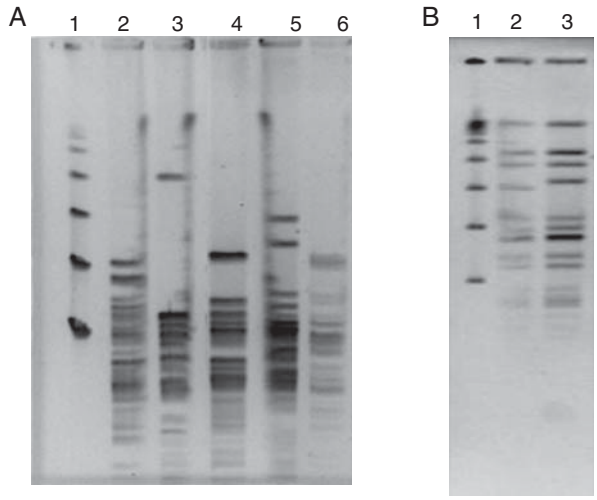

FIGURE 1. Fingerprinting profile (pulsed-field gel electrophoresis) of the strains *Lactobacillus fermentum* LF10 (DSM 19187), *L. fermentum* LF11 (DSM 19188), and *Lactobacillus acidophilus* LA02 (DSM 21717). A, *L. fermentum* LF10 and *L. fermentum* LF11 (enzyme *Xba*I). (1) Electrophoretic marker, Sigma 50 to 1000 kb; (2) comparison strain, *L. fermentum* ID 1323; (3) comparison strain, *L. fermentum* LF5 (CNCM I-789); (4) sample strain, *L. fermentum* LF10; (5) sample strain, *L. fermentum* LF11; (6) comparison strain, *L. fermentum* DSM 20052. B, *L. acidophilus* LA02 (enzyme *Sma*I). (1) Electrophoretic marker, Sigma 50 to 1000 kb; (2) comparison strain, *L. acidophilus* DSM 20079; (3) sample strain, *L. acidophilus* LA02.

beginning of incubation, the ability of a probiotic bacterium to antagonize a specific target microorganism could also be entirely exerted by a freeze-dried culture, the form commonly used in commercial products. The results are expressed as percentages of inhibition of *Candida* growth after 24 and 48 hours of incubation and as inhibition extent expressed as $\log_{10}$. A comparison between viable cell counts of *Candida* after 24 or 48 hours and time 0 inoculum is included as well (Table 1).

L. acidophilus LA02 was included as a negative control, which is a probiotic able to exert only unspecific inhibitory effects exclusively mediated by the organic acids produced and the consequent pH lowering. As *L. acidophilus* species has a homofermentative metabolism, the sole organic acid produced is lactic.

The most relevant differences were recorded after 24 hours, with *L. acidophilus* LA02 generally able to inhibit the growth of only 56.7% to 93.3% of *Candida* cells compared with samples of yeast cultured alone. *L. fermentum* LF10 and LF11 were able to inhibit not $<98\%$ of *Candida* at 24 hours, with the average values around 99.8%.

The differences are considerably more appreciable if expressed as base-10 logarithms, ranging from 1.70 to 4.19 after 24 hours for the 2 *L. fermentum* compared with 0.37 to 1.17 recorded with *L. acidophilus* LA02. Moreover, at 24 hours, a significant *Candida* growth was observed when cocultured with *L. acidophilus* LA02 (variation vs. time 0 from 83.9 to 2,280 times), whereas no significant growth compared with inoculum was recorded with any of the *L. fermentum* used. In fact, variations ranged from 0.10, meaning a 10-time decrease, to 61.0 recorded with *C. glabrata* ATCC 2001, apparently the most resistant yeast strain.

After 48 hours, *L. acidophilus* LA02 was able to cause a slight mortality of only *C. krusei* ATCC 6258 (variation vs. time 0 = 0.8), whereas all the other *Candida* strains were able to grow (variation vs. time 0 ranging from 24.9 to 1170). In any case, *Candida* viable cell counts were always higher at 24 hours compared with 48 hours, meaning that the lactic acid produced by *L. acidophilus* LA02 needs a certain period of time to unspecifically restrict yeast growth.

In contrast, both *L. fermentum* probiotics showed a very strong inhibition of all *Candida* after 48 hours. In many samples, <10 CFU/mL of broth were retrieved. *C. parapsilosis* ATCC 22019 seemed to be the relatively most resistant strain, even if 3.5 base-10 logarithms of inhibition were recorded with both *L. fermentum* compared with 1.46 mediated by *L. acidophilus*.

Figure 2 reports the growth kinetics of *C. albicans* ATCC 10231 cultured either alone or in the presence of individual probiotics used as freeze-dried samples.

Results from freeze-dried cultures, assessed using *C. albicans* ATCC 10231 as indicator of activity, are similar to values recorded with fresh broth cultures, therefore suggesting that the physical status of the bacterial population is not able to quantitatively influence its ability to exert a strong protective barrier effect against *Candida*. The higher inoculum of *C. albicans* ATCC 10231 compared with the previous experiment made the difference in inhibition extents even more significant between both *L. fermentum* and *L. acidophilus*.

Results of the In Vivo Human Efficacy Study

All female patients completed the treatment, meaning there were no drop-outs, thus confirming the very high tolerability profile of the product ActiCand 30. In the examination at the end of the first 4 weeks of product application, performed by both microscopic assessment and yeast culture, a complete healing and disappearance of *Candida* infection was found in 26 patients out of 30 (corresponding to 86.6%, $P < 0.001$) (Tables 2 and 3). Furthermore, no recurrence occurred or was reported after the first week of treatment with 1 tablet a day, thus suggesting that the application of 1 tablet every 3 nights for a further 3-week period is sufficient to completely prevent relapses when the probiotics of ActiCand 30 are already integrated in the vaginal microbiota.

At the end of the second month, during which patients were directed to apply 1 tablet every 7 days, a recurrence episode was recorded, albeit not particularly serious, in only 3 out of 26 patients (11.5%, $P = 0.083$) who were found to have fully healed at the end of the first month of treatment. In any case, the statistical comparison between results at the end of the second month and time 0 is still significant ($P < 0.001$) (Table 3).

The *Candida* species responsible for VVC diagnosed at enrollment were identified (Table 4).

DISCUSSION

The in vitro inhibitory results strongly suggested that the mechanism of action of the *L. fermentum* probiotics is at least partially based on specific molecules other than organic acids, such as bacteriocins, extracellular proteins, or hydrogen peroxide. In other words, acidity alone did not inhibit the growth of individual *Candida* strains to the same extent as *L. fermentum* LF10 and LF11. The major difference was that *L. acidophilus* LA02 was only able to limit the

TABLE 1. Inhibitory Activity of *Lactobacillus fermentum* LF10, *L. fermentum* LF11, and *Lactobacillus acidophilus* LA02 Toward 5 *Candida* Yeasts (A) or *Candida albicans* ATCC 10231 (B)

Time	Sample	*C. albicans* ATCC 10231	*Candida parapsilosis* ATCC 22019	*Candida krusei* ATCC 6258	*C. albicans* ATCC 90028	*Candida glabrata* ATCC 2001
(A) Fresh broth cultures						
Time 0 (inoculum)	Yeast alone	**2450**	**3100**	**1870**	**1300**	**1000**
24 h	Yeast alone	**3,860,000**	**600,000**	**660,000**	**4800,000**	**12,000,000**
	Yeast + LF10	**250**	**2700**	**2400**	**4000**	**12,600**
	% growth inhibition*	99.994	99.550	99.636	99.917	99.895
	Inhibition as $\log_{10}$†	4.19	2.35	2.44	3.08	2.98
	Variation vs. time 0‡	0.10	0.87	1.28	3.08	12.60
	Yeast + LF11	**520**	**12,000**	**600**	**4480**	**61,000**
	% growth inhibition*	99.987	98.000	99.909	99.907	99.492
	Inhibition as $\log_{10}$†	3.87	1.70	3.04	3.03	2.29
	Variation vs. time 0‡	0.21	3.87	0.32	3.45	61.00
	Yeast + LA02	**260,000**	**260,000**	**280,000**	**470,000**	**2,280,000**
	% growth inhibition*	93.264	56.667	57.576	90.208	81.000
	Inhibition as $\log_{10}$†	1.17	0.36	0.37	1.01	0.72
	Variation vs. time 0‡	106.12	83.87	149.73	361.54	2280.00
48 h	Yeast alone	**4,500,000**	**3,500,000**	**2,700,000**	**8,800,000**	**34,000,000**
	Yeast + LF10	**7**	**1100**	**900**	**20**	**137**
	% growth inhibition*	100.000	99.969	99.967	100.000	100.000
	Inhibition as $\log_{10}$†	5.81	3.50	3.48	5.64	5.39
	Variation vs. time 0‡	0.003	0.355	0.481	0.015	0.137
	Yeast + LF11	**0**	**1100**	**3**	**9**	**12**
	% growth inhibition*	100.000	99.969	100.000	100.000	100.000
	Inhibition as $\log_{10}$†	6.65	3.50	5.95	5.99	6.45
	Variation vs. time 0‡	0.000	0.355	0.002	0.007	0.012
	Yeast + LA02	**61,000**	**120,000**	**1500**	**59,000**	**1,170,000**
	% growth inhibition*	98.644	96.571	99.944	99.330	96.559
	Inhibition as $\log_{10}$†	1.87	1.46	3.26	2.17	1.46
	Variation vs. time 0‡	24.90	38.71	0.80	45.38	1170.00
(B) Freeze-dried cultures						
Time 0 (inoculum)	Yeast alone	**50,000**				
24 h	Yeast alone	**8,600,000**				
	Yeast + LF10	**310**				
	% growth inhibition*	99.996				
	Inhibition as $\log_{10}$†	4.44				
	Variation vs. time 0‡	0.006				
	Yeast + LF11	**60**				
	% growth inhibition*	99.999				
	Inhibition as $\log_{10}$†	5.16				
	Variation vs. time 0‡	0.001				
	Yeast + LA02	**270,000**				

TABLE 1. (*continued*)

Time	Sample	*C. albicans* ATCC 10231	*Candida parapsilosis* ATCC 22019	*Candida krusei* ATCC 6258	*C. albicans* ATCC 90028	*Candida glabrata* ATCC 2001
	% growth inhibition*	96.860				
	Inhibition as log_{10}†	1.50				
	Variation vs. time 0‡	5.400				
48 h	Yeast alone	**110,000,000**				
	Yeast + LF10	**1**				
	% growth inhibition*	100.000				
	Inhibition as log_{10}†	8.04				
	Variation vs. time 0‡	0.000				
	Yeast + LF11	**0**				
	% growth inhibition*	100.000				
	Inhibition as log_{10}†	8.04				
	Variation vs. time 0‡	0.000				
	Yeast + LA02	**120,000**				
	% growth inhibition*	99.891				
	Inhibition as log_{10}†	2.96				
	Variation vs. time 0‡	2.400				

Probiotics were used either as fresh broth cultures or as freeze-dried samples. Viable cell counts of *Candida* (bold values) are expressed as CFU/mL.

*Percentage of *Candida* cells unable to grow in the presence of a probiotic strain after either 24 or 48 hours of incubation. The comparison was made with the number of *Candida* cells cultured alone after the same period of time.

†Number of base-10 logarithms of inhibition mediated by each probiotic after either 24 or 48 hours of incubation. Values < 1 show inhibition of < 1 order of magnitude, whereas values > 1 mean a decrease in the number of *Candida* cells at least > 10 times. The comparison was made with the number of *Candida* cells grown alone after the same period of time.

‡Number of times of change with respect to time 0 (inoculum). Values < 1 show a decrease in the number of yeasts, whereas numbers > 1 mean a growth of the *Candida* tested compared with its time 0 inoculum.

growth of *Candida* in coculture compared with the yeast alone, whereas both *L. fermentum* not only prevented any significant growth of *Candida* at 24 hours, but also exerted a noticeable microcidal activity at 48 hours.

One of the 2 *L. fermentum* strains was chosen to formulate the new vaginal probiotic product ActiCand 30 together with *L. acidophilus* LA02, used to more extensively restore the vaginal Döderlein complex.[36] In defining the formulation of a vaginal product, it should always be taken into account that the way used to deliver probiotics to the vaginal environment is a crucial point.

The results collected from this human efficacy pilot study demonstrate the effectiveness of ActiCand 30 in the establishment and maintenance of a protective barrier effect against vaginal *Candida*, well known as the causative agent of even severe vaginitis, able to relapse and heavily compromise the overall quality of life of the women affected.

The results showed that most yeast infections were caused by the *C. albicans* species (80.0%), and this is consistent with the findings reported in literature. Two out of 3 relapses recorded in the second month of the study protocol were to be ascribed to *C. albicans* and the remaining to the *C. krusei* species, even if this was not a real relapse of the same preexistent infection. At enrollment, no positive *C. krusei* yeast culture was found. The woman who experienced a *C. krusei* positive result at d_{56} was diagnosed with a *C. albicans* infection at d_0. This infection was found to have fully healed at the end of the first month (d_{28}) of treatment; therefore, this should be more properly regarded as a new infection case rather than a classic recurrence. In any case, the number of *C. krusei* cells enumerated on YGC agar was < 10 and also the visual examination of the vaginal mucosa suggested a very mild infection.

The compliance and tolerability profile were very good, as no drop-out was recorded during the entire study protocol.

ActiCand 30 was specifically formulated in slow release effervescent tablets to rapidly create an anaerobic vaginal microenvironment after application, able to be reasonably maintained for at least 40 to 60 minutes, because the disaggregation time of a single tablet is around 30 minutes. Anaerobiosis is well known to encourage the colonization and proliferation of lactobacilli and Döderlein endogenous biota in general, while restricting the growth of *Candida* spp. in light of their respirer yeast feature.

In contrast, a longer time is needed for the establishment of the protective barrier effect exerted by the 2 probiotics *L. fermentum* LF10 and *L. acidophilus* LA02.

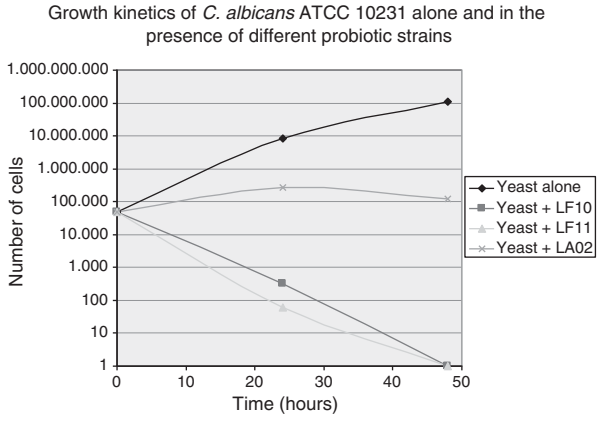

FIGURE 2. Kinetics of growth of *Candida albicans* ATCC 10231 incubated either alone or in the presence of individual *Lactobacillus fermentum* LF10, *L. fermentum* LF11, or *Lactobacillus acidophilus* LA02.

In summary, 2 specific chronologically distinct actions are mediated by ActiCand 30:

- Short-term barrier effect exerted by the CO_2 released during the slow disaggregation of the tablet. CO_2 is also able to partly lower the vaginal pH after hydration and subsequent formation of carbonic acid, which is a diprotic acid that may dissociate into bicarbonate ion, HCO_3^-, and H^+;
- Long-term protective barrier effect guaranteed by the vaginal microbiota colonization by the 2 probiotics, able to restore the Döderlein complex, habitually compromised in case of candidiasis. These beneficial bacteria are able to mediate both a mechanical action (competition for adhesion sites to the mucosa) and a metabolic activity consisting of the production of organic acids and other more specific molecules, especially bacteriocins.[37]

Also of particular interest is the product's ability to prevent recurrences, a very common issue in VVC. The overall effectiveness of ActiCand 30 could be regarded as very similar to most commercial products traditionally and historically used to treat or prevent vulvovaginitis by *Candida*. Furthermore, this product has the valuable advantage of the lack of any adverse side-effect, because lactobacilli are an integral and desirable part of the endogenous vaginal microbiota.

Further assessments will be needed to completely identify and characterize the molecules at the basis of the mechanism of action of *L. fermentum* LF10 and LF11 toward *Candida* yeasts. In any case, this study further strengthens the evidence supporting the use of specific probiotic strains with well-demonstrated activities for the

TABLE 2. Incidence of *Candida* Infections at the Beginning of the Study (Time 0), After 4 Weeks of Treatment (Time 28), and at the End of the Second Month of the Study Protocol (Time 56)

			Infection by *Candida*		
Subjects	**Age**	**Menopause**	**Time 0**	**Time 28**	**Time 56**
A.R.	45	No	X	—	—
L.C.	36	No	X		X
M.T.	51	No	X	—	—
L.M.	58	Yes	X	—	—
R.R.	23	No	X	X	X
L.C.	32	No	X	—	—
T.T.	38	No	X	—	—
M.R.	61	Yes	X	—	—
L.S.	26	No	X	—	—
M.S.	28	No	X	—	X
O.R.	36	No	X	—	—
P.M.	44	No	X	—	—
S.D.	40	No	X	X	X
A.B.	49	Yes	X	—	—
D.U.	52	Yes	X	—	—
M.F.	32	No	X	X	X
V.L.	30	No	X	—	—
V.M.	38	No	X	—	—
P.L.	55	Yes	X	—	—
S.A.	27	No	X	—	—
M.B.	33	No	X	—	—
S.R.	36	No	X	—	—
T.I.	39	No	X	—	—
A.B.	64	Yes	X	—	X
J.P.	54	Yes	X	—	—
R.F.	43	No	X	—	—
E.S.	30	No	X	—	—
E.T.	53	Yes	X	—	—
F.B.	32	No	X	X	X
F.G.	41	No	X	—	—

TABLE 3. Overview of Data and Statistical Analysis

Parameters	**Time 0**	**Time 28**	**p (T28 vs. T0)**	**Time 56**	**p (T56 vs. T0)**	**p (T56 vs. T28)**
Total women with infection	30	4	< 0.001	7	< 0.001	—
Total women without infection	0	26	—	23	—	—
Percentage of healing	—	86.67%	—	76.67%	—	—
Total women with recurrences	—	0	—	3	—	0.083
Percentage of recurrences	—	—	—	11.54%	—	—

TABLE 4. Species Identification of Vaginal Yeast Isolates. The Percentages of Individual *Candida* Species at Time 28 and 56 Refer to the Total Number of Women With a Positive Yeast Culture

***Candida* Species**	**Time 0 N = 30**	**Time 28 N = 4**	**Time 56 N = 7**
Candida albicans	24 (80.0%)	1 (25.0%)	3 (42.8%)
Candida glabrata	2 (6.7%)	1 (25.0%)	1 (14.3%)
Candida papapsilosis	1 (3.3%)	0	0
Candida tropicalis	1 (3.3%)	1 (25.0%)	1 (14.3%)
Candida krusei	0	0	1 (14.3%)
Other Candida species	2 (6.7%)	1 (25.0%)	1 (14.3%)
Organisms other than *Candida*	0	0	0
Total positive *Candida* cultures	30 (100.0%)	4 (100.0%)	7 (100.0%)

creation and maintenance of a vaginal microenvironment that do not encourage the establishment, propagation, or persistence of an infection caused by *Candida*, not only in relation to *C. albicans* species, but also *C. glabrata*, *C. parapsilosis*, and *C. krusei*.

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