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(71) Applicant: **PROBIOTICAL S.P.A.** [IT/IT]; Via E. Mattei, 3, 28100 Novara NO (IT).

(72) Inventor: **MOGNA, Giovanni**; c/o Probiotical S.P.A., Via E. Mattei, 3, 28100 Novara (IT).

(74) Agents: **HOFFMANN EITLE S.R.L.** et al.; Piazza Sigmund Freud 1, Torre 2, Piano 22, 20154 Milano (IT).

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(54) Title: LACTIC ACID BACTERIAL COMPOSITION FOR THE SIMULTANEOUS TREATMENT OF VAGINAL INFECTIONS OF FUNGAL AND BACTERIAL ORIGIN

(57) Abstract: The present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement (briefly, the composition (s) of the present invention) based on lactic acid bacteria belonging to the species Lactobacillus fermentum for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases of fungal and bacterial origin. Specifically, the present invention relates to a said composition comprising pharmaceutical- and/or food-grade excipients and a mixture, which comprises or, alternatively, consists of at least a strain of bacteria belonging to the species Lactobacillus fermentum for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis.

DESCRIPTION of the invention entitled: ***Lactic acid bacterial composition for the simultaneous treatment of vaginal infections of fungal and bacterial origin.***

The present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement (briefly, the composition(s) of the present invention) based on lactic acid bacteria belonging to the species *Lactobacillus fermentum* for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases of fungal and bacterial origin. Specifically, the present invention relates to a said composition comprising pharmaceutical- and/or food-grade excipients and a mixture, which comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis.

The composition of the intestinal and urogenital microflora is known to represent a critical point for women's health and wellbeing. The vaginal ecosystem consists of epithelial cells lining the vagina and uterus, gland cells secreting in the lumen of the organ and a complex bacterial flora represented by different species of microorganisms.

Such microorganisms are able to fermenting the glycogen deriving from the decomposition of parabasal cells of the eutrophic vaginal mucosa, resulting in the production of lactic acid, the final effect of which is the establishment and maintenance of an acidic vaginal environment (with pH values of approximately 4-4.5 under physiological conditions).

Hydrogen ions (H^+) deriving from lactic acid contribute to the formation of hydrogen peroxide. Such a peroxide molecule is toxic for a large number of bacterial species lacking the enzyme catalase. In vaginal secretions, concentrations of about 0.75-5 µg/ml are readily reached, which are more than sufficient for the toxic effect to be exerted.

The combined action of hydrogen peroxide, uterine peroxidase (produced by the cervix and endometrium) and chloride (Cl^-) and iodide (I^-) ions limits the bacterial growth even by directly activating polymorphonuclear cells, which exert a bactericidal action in the epithelial intercellular spaces.

Due to various exogenous and endogenous factors, such as the use of antibiotics, stress conditions, hormonal modulations related to pregnancy, menstrual cycle and/or taking high estrogen concentrations, an unbalance of the vaginal ecosystem often occurs in women. An alteration in the balance of the vaginal ecosystem leads to a prevalence of microorganisms such as *Candida albicans* and *glabrata* and/or *Gardnerella vaginalis*, which cause candidiasis, vaginitis, vulvovaginitis and bacterial vaginosis.

It is known that approximately 75% of women suffered or will suffer, at some point in their life, from at least an episode of vulvovaginal mycosis, due to a fungus (yeast) called *Candida albicans* (Candida vaginitis or

vulvovaginitis) in 90% of cases. Moreover, it has to be noted that a relapse, namely a further infectious event, occurs in about 40-50% of women.

Furthermore, another important aspect of vaginal infections concerns bacterial vaginosis. A bacterial vaginosis is known to be a vaginal inflammation of bacterial origin and is the most common cause of vaginal disorders in childbearing and menopausal women. Bacterial vaginosis is caused by an alteration in the balance of the vaginal microflora, with abnormal development of commensal germs, which, following to an alteration of the vaginal ecosystem, turn into pathogens due to many causes. The main agent responsible for most of the episodes of bacterial vaginosis, almost 80% of cases, is *Gardnerella vaginalis*. Bacterial vaginosis can be treated with antibiotics such as for example metronidazole or clindamycin. However the bacterial, as well as the fungal vaginosis, can also relapse even after therapy. Therefore, the antibiotic treatment does not represent in any way a definitive treatment, is ineffective in avoiding one or more relapses, nor is a treatment suitable for all the categories of patients.

At present, epidemiological data show that vaginal infections affect more than one billion of women per year worldwide, with serious socio-economic consequences. Furthermore, vaginosis has been related to preterm delivery and a high incidence of necrotizing enterocolitis (NEC). A link between autism and perinatal inflammation has been suggested.

The use of antibiotics during pregnancy has been associated with the onset of asthma.

Vulvovaginal candidiasis (VVC) is usually caused by *C. albicans* but it can also be due to other species of *C. albicans* or yeasts. Typical VVC symptoms comprise itching, vaginal irritation, dyspareunia, external dysuria and abnormal vaginal secretions. None of these symptoms is VVC-specific.

C. glabrata is a pathogenic yeast with various and unique biological characteristics. It could be isolated from the skin and urine, and represents one of the most common yeasts found on mucous membranes. It is considered as an opportunistic pathogen causing surface and systemic infections, in particular in immunocompromised patients. In Europe, the percentage of overall resistance to fluconazole of *C. glabrata* strains is 16.5%.

C. parapsilosis is an exogenous pathogen. It can lead to sepsis, onychomycosis and dermatomycosis, is found also in mucosae, and has biochemical characteristics different from the other species.

C. tropicalis is the most common pathogen of the genus Candida. It is found in mixed cultures along with *C. albicans* and is very resistant to antifungal therapies. This pathogen can proliferate on mucous membranes and can be observed even in the absence of specific signs of a disease.

C. krusei is a fungus naturally resistant to fluconazole, and can be isolated from beer, milk and derivatives, and skin, saliva and feces from animals. It is related to some forms of infantile diarrhea and occasionally to systemic diseases. Such a fungus is able to colonizing the intestine, the respiratory and urinary tracts of patients with granulocytopenia.

Bacterial vaginosis (BV) is a clinical polymicrobial syndrome due to the replacement of the normal lactobacilli capable to produce hydrogen peroxide by high concentrations of anaerobic bacteria (e.g. the species *Prevotella* and *Mobiluncucus*), ureaplasma, mycoplasma and several and annoying kinds of anaerobic organisms. Among women in need of treatment, BV is the main cause of vaginal secretions and odor. However, in a study conducted in USA, most of women with BV were found to be asymptomatic. The percentage of women, which experienced at least an episode of BV ranges between 5 and 70%. BV is characterized by the *Gardnerella vaginalis* infection in 80% of reported cases.

After a gynecological diagnosis subsequent to a laboratory fungal analysis, an antibiotic and/or fungicidal therapy is generally chosen, which usually leads to good results in the short term, but is unable to prevent recurrent infections, due to the increasing resistance of pathogens. Furthermore, not all the subjects in need of treatment are able to take and tolerate an antibiotic or fungicidal therapy.

Miconazole and fluconazole represent the most used active ingredients for treating candidiasis: miconazole is mainly used topically for infections due to *C. albicans* and species other than *C. albicans*. Among triazoles, fluconazole is the molecule of choice for the treatment of candidiasis, but its efficacy is variable: in fact from 29% to 43% of patients with systemic infections obtain no satisfactory results with these molecules. In addition, these molecules have a highly variable effectiveness against different species of yeasts.

It is also known the use of microorganisms, such as for example lactic acid bacteria, able to restoring a proper composition of the vaginal microflora. Lactic acid bacteria can be used as active ingredient in a composition for vaginal use, which can be for example in a solid form as an oral tablet, lozenge, granules or powder or solid form as a vaginal tablet or in a liquid form as a vaginal douche, solution or gel.

However, thus far, there is still a need for having a fast and effective treatment against vaginal infections, disorders or diseases independent from a gynecologic diagnosis and microbiological analyses, in order to achieve rapid and beneficial results from the treatment of a bacterial vaginosis and/or Candida vaginitis. Basically, it would be very useful to provide a treatment, which, for its application, does not need a diagnosis, which would imply for the patient to be subjected to medical examinations and sample withdrawals, is devoid of side effects typical of the commonly used antibiotics/antifungal drugs, avoids the development of cross-resistance to antibiotics and is highly tolerable and effective.

Specifically, there is still a need for having a composition for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases of fungal and bacterial origin which is (i) effective and/or (ii) easy to prepare and/or (iii) able to definitively act on the balance of the vaginal ecosystem and/or (iv) effective in avoiding one or more relapses and suitable for all the categories of patients, said composition having a broad-spectrum activity against pathogenic microorganisms among which, but not limited to, yeasts of the genus *Candida*, responsible for fungal candidiasis, vaginitis and vulvovaginitis, and the bacterium *Gardnerella vaginalis*, which accounts for 80% of cases of bacterial vaginosis.

Furthermore, there is still a need for having a composition for vaginal and oral use for the simultaneous treatment of vaginal infections, disorders or diseases of fungal (namely, due to fungi and/or yeasts) and bacterial origin, which represents a valid alternative to an antibiotic and/or fungicidal therapy and, at the same time, represents an effective treatment relative to the known administration forms against both *Candida* and *Gardnerella vaginalis* infections.

The Applicant, after an extended and demanding research and development activity, developed a composition for vaginal or oral use based on lactic acid bacteria being able to effectively solve the common problems of the known technique.

It is an object of the present invention a composition for vaginal or oral use, as set forth in the appended independent claim.

Other preferred embodiments of the present invention are set forth in the appended dependent claims.

Graph 1 relates to an illustrative histogram of the effect of the co-growth of lactobacilli and *C. albicans* in MRS.

Graph 2 relates to histograms representing the mortality of different species of Candida co-grown with LF5.

Graph 3 relates to histograms representing the mortality of different species of Candida co-grown with LF5.

Il **Graph 4** relates to histograms representing the mortality of different species of Candida co-grown with LF5.

Figure 1 relates to the action exerted by LF5 on C. albicans ATCC 90028 during the co-growth of the two microorganisms.

Figure 2 relates to the inhibition halos of *C. albicans* ATCC 90028 on agar medium induced by a broth culture of the strain LF5: disc technique.

Figure 3 relates to the inhibition halos of the growth of *C. albicans* ATCC 90028 on agar medium induced by the freeze-dried concentrate of the supernatant from the disc and spot techniques.

Figures 4, 5 and 6 relate to the mechanism of action of the strain LF5 against *C. albicans.*

Graph 5 relates to the inhibition of C. albicans ATCC 90028: Graphs of overall data, at experiment 1 and experiment 2.

Table 11 relates to experiments on strains of bacteria *L. Fermentum* showing the MICs at 24 and 48 hours.

Figure 7 shows the results of the clinical study for the efficacy and tolerability of LF5 compared with placebo in patients with *Candida albicans*.

Figure 8a shows the trend of the overall average number of symptoms and signs (+ESM) related to the fungal vaginal colonization, during and after the treatment with LF5 or miconazole.

Figure 8b shows the trend of the total average intensity of symptoms and signs (+ESM) related to the fungal vaginal colonization, during and after the treatment with LF5 or miconazole.

Figure 9 shows the activity of different strains of *Lactobacillus fermentum* against several species of *Candida*.

Figure 10 shows the activity of *Lactobacillus fermentum* LF5 against several species of *Candida*.

The Applicant carried out a long and intense experimental research activity, during which tested a first group of bacterial strains belonging to the species below:

- *L. plantarum*, such as for example the strain of bacteria *L. plantarum* (LP 01) LMG P-21021;
- *L. pentosus*, such as for example the strain of bacteria *L. pentosus* (LPS 01) DSM 21980;
- *L. delbrueckii ssp. delbrueckii*, such as for example the strain of bacteria *L. delbrueckii ssp. delbrueckii* (DSMZ 20074 LDD01) DSM 22106;
- *L. rhamnosus*, such as for example the strain of bacteria *L. rhamnosus* (LR 06) DSM 21981;
- *B. breve*, such as for example the strain *B. breve* (BR 03) DSM 16604.

Said bacterial strains were tested in order to assess the inhibition data on *Gardnerella vaginalis* (responsible for 80% of cases of bacterial vaginosis in women) as well as the activity and efficacy thereof against *Candida albicans*. The results of said inhibition tests were negative for the pathogen *Gardnerella vaginalis*. The tests came back negative, since none of the tested strains was able to inhibit the pathogen *Gardnerella vaginalis*.

The strain of the pathogen *Gardnerella vaginalis* being used in the tests of the present invention is the type *Gardnerella vaginalis* ATCC 14018, also referred to as *Haemophilus vaginalis*, as reported below: *Gardnerella vaginalis* ATCC® Number: 14018™, Preceptrol® Culture. Organism: *Gardnerella vaginalis* (Gardner and Dukes) Greenwood and Pickett deposited as *Haemophilus vaginalis* Gardner and Dukes. ATCC Medium: 814 GC Medium.

The Applicant then tested a second group of vaginal bacterial strains belonging to the species below:

(i) *L. crispatus*, such as for example the strain of bacteria *L. crispatus* CRL 1266 ID1626 DSM 24439;
(ii) *L. crispatus*, such as for example the strain of bacteria *L. crispatus* CRL 1251 ID1606 DSM 24438;
(iii) *L. paracasei*, such as for example the strain of bacteria *L. paracasei* LPC 08 ID1696 DSM 21718;
(iv) *L. paracasei*, such as for example the strain of bacteria *L. paracasei* CRL 1289 ID1608 DSM 24440;
(v) *L. fermentum*, such as for example the strain of bacteria *L. fermentum* LF 11 ID1639 DSM 19188.

Table 1

	Amount of fresh MRS

	0	0.5	1	2	4
G. vaginalis	0.497	1.251	1.534	1.811	1.807

	Amount of supernatant				
Neutralized supernatant of the strain:		0.5	1	2	4
L. crispatus CRL 1266 ID 1626 DSM 24439		0.505	0.511	0.673	1.035
L. crispatus CRL 1251 ID 1605 DSM 24438		0.531	0.444	0.422	0.491
L. paracasei LPC08 ID 1696 DSM 21718		0.653	1.130	1.538	1.724
L. paracasei CRL 1289 ID 1608 DSM 24440		/	/	1.476	1.774
L. fermentum LF 11 ID 1639 DSM 19188		/	0.339	0.302	/
Ecocillin INOCULUM		0.794	1.074	1.391	1.690

Table 1 reports the results of the inhibition test (Analytical method *infra*) of *G. vaginalis* by the strains of vaginal bacteria mentioned above from (i) to (v).

Results in Table 1 refer to the optical density, which was detected at the wavelength OD_{600} after 24 hours of microaerophilic growth. The test was performed with a starting inoculum at 2% from fresh broth culture in TH Broth. It was also observed that adding the MRS medium increased the growth ability of the pathogen.

Then, the Applicant tested a third group of strains of bacteria belonging to the species *Lactobacillus fermentum*, in order to assess the inhibition data on *Gardnerella vaginalis* (responsible for 80% of cases of bacterial vaginosis in women) as well as the activity and efficacy thereof against *Candida albicans* and other yeasts of the genus *Candida*.

The Applicant only selected the strains of bacteria belonging to the species *L. fermentum*, which shown to be active and effective against both the pathogens *Candida albicans* and *Gardnerella vaginalis*.

The results of absorbance OD_{600}, at 24 hours and 48 hours, are reported in Table 2 and Table 3, respectively.

Table 2: inhibition test (Analytical method *infra*)

	Amount of fresh MRS		
24 hours	0.5	1	2
Gardnerella vaginalis	1.379	1.542	1.567

Strain	Amount of supernatant		
	0.5	1	2
No. 1 *L. Fermentum* LF 5 ID 686 DSM 32277	0.745	0.608	0.703
No. 2 *L. Fermentum* LF 5 ID 686 CNCM I-789	0.745	0.608	0.703
No. 3 *L. Fermentum* LF 06 ID 1456	0.727	0.541	0.591
No. 4 *L. Fermentum* LF 07 ID 1459	0.677	0.530	0.589
No. 5 *L. Fermentum* LF 08 ID 1460	0.681	0.583	0.717
No. 6 *L. Fermentum* LF 09 ID 1462	0.715	0.551	0.615
No. 7 *L. Fermentum* LF 10 ID	0.736	0.610	0.682

1637			
No. 8 *L. Fermentum* LF 11 ID 1639	0.683	0.725	0.688
No. 9 *L. Fermentum DPPMA* 114 ID 1757	0.783	0.735	0.669
No. 10 *L. Fermentum* Lb2 ID 1753	0.827	0.884	0.761
No. 11 *L. Fermentum* LF 15 ID 1852	0.765	0.628	0.723
No. 12 *L. Fermentum* LF 16 ID 1853	0.716	0.580	0.652
No. 13 *L. Fermentum* LF 18 ID 1897	0.673	0.715	0.668
No. 14 *L. Fermentum* LF 25 ID 1956	0.775	0.648	0.733

Table 3: inhibition test (Analytical method *infra*)

	Amount of fresh MRS		
48 hours	0.5	1	2
Gardnerella vaginalis	1.384	1,554	1,562

Strain	Amount of supernatant		
	0.5	1	2
No. 1 *L. Fermentum* LF 5 ID 686 DSM 32277	0.574	0,337	0,233

No. 2 *L. Fermentum* LF 5 ID 686 CNCM I-789	0.574	0.337	0.233
No. 3 *L. Fermentum* LF 06 ID 1456	0.617	0.463	0.435
No. 4 *L. Fermentum* LF 07 ID 1459	0.489	0.474	0.379
No. 5 *L. Fermentum* LF 08 ID 1460	0.510	0.532	0.372
No. 6 *L. Fermentum* LF 09 ID 1462	0.797	0.759	0.374
No. 7 *L. Fermentum* LF 10 ID 1637	0.618	0.696	0.447
No. 8 *L. Fermentum* LF 11 ID 1639	0.466	0.488	0.357
No. 9 *L. Fermentum DPPMA* 114 ID 1757	0.499	0.588	0.473
No. 10 *L. Fermentum* Lb2 ID 1753	0.532	0.601	0.583
No. 11 *L. Fermentum* LF 15 ID 1852	0.594	0.367	0.263
No. 12 *L. Fermentum* LF 16 ID 1853	0.588	0.676	0.427

No. 13 *L. Fermentum* LF 18 ID 1897	0.496	0.498	0.377
No. 14 *L. Fermentum* LF 25 ID 1956	0.601	0.377	0.273

Therefore, it is an object of the present invention a pharmaceutical composition or a composition for a medical device or a composition for a food supplement or a composition for a food product (briefly, the composition(s) of the present invention) comprising pharmaceutical- and/or food-grade excipients and a mixture consisting of or, alternatively, comprising at least a strain of bacteria belonging to the species *Lactobacillus fermentum*; said composition being for vaginal or oral use for the treatment of vaginal infections, disorders or diseases selected from candidiasis, vaginitis, vulvovaginitis and/or bacterial vaginosis.

An embodiment of the present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement or a composition for a food product (briefly, the composition(s) of the present invention) comprising pharmaceutical- and/or food-grade excipients and a mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the bacterial strains from 1 to 14 (listed in Table 4); said composition being for vaginal or oral use for the treatment of vaginal infections, disorders or diseases selected from candidiasis, vaginitis, vulvovaginitis and/or bacterial vaginosis.

Table 4

List of tested strains	Abbreviation	ID	Deposit	Depositor
No. 1 *L. fermentum*	LF 05	686	DSM 32277	Probiotical SpA
No. 2 *L. fermentum*	LF 05	686	CNCM I-789	Probiotical SpA
No. 3 *L. fermentum*	LF 06	1456	DSM 18295	Anidral Srl
No. 4 *L. fermentum*	LF 07	1459	DSM 18296	Anidral Srl
No. 5 *L. fermentum*	LF 08	1460	DSM 18297	Anidral Srl

No. 6 *L. fermentum*	LF 09	1462	DSM 18298	Anidral Srl
No. 7 *L. fermentum*	LF 10	1637	DSM 19187	Anidral Srl
No. 8 *L. fermentum*	LF 11	1639	DSM 19188	Anidral Srl
No. 9 *L. fermentum*	DPPMA 114	1757	DSMZ 23757	Probiotical SpA
No. 10 *L. fermentum*	Lb2	1753	DSM 16143	Probiotical SpA
No. 11 *L. fermentum*	LF 15	1852	DSM 26955	Probiotical SpA
No. 12 *L. fermentum*	LF16	1853	DSM 26956	Probiotical SpA
No. 13 *L. fermentum*	LF18	1897	DSM 29197	Probiotical SpA
No. 14 *L. fermentum*	LF 25	1956	DSM 32275	Probiotical SpA

An embodiment of the present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement or a composition for a food product (briefly, the composition(s) of the present invention) comprising pharmaceutical- and/or food-grade excipients and a mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum*; said composition being for vaginal or oral use for the treatment of a vaginal infection, disorder or disease selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis caused by at least a pathogen selected from the group comprising: *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis, Gardnerella vaginalis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Herpes simplex* and *Hemophilus Ducreyi*e, wherein the bacteria belong to the strain *Lactobacillus fermentum (*LF05) with deposit number DSM 32277 or CNCM I-789 (depositor Probiotical SpA).

A preferred embodiment of the present invention relates to the pharmaceutical composition or composition for a medical device or composition for a food supplement or composition for a food product (briefly, the composition(s) of the present invention), as defined above, comprising pharmaceutical- and/or food-grade excipients and a mixture which, in addition to the bacteria belonging to the strain *Lactobacillus fermentum*

*(*LF05) with deposit number DSM 32277 or CNCM I-789 (depositor Probiotical SpA), comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 7, 8, 11, 12, 13 and 14 (listed in Table 4); said composition being for vaginal or oral use for the treatment of vaginal infections, disorders or diseases selected from candidiasis, vaginitis, vulvovaginitis or vaginosis. Advantageously, said at least a strain of bacteria belonging to the species *Lactobacillus fermentum* is selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 8, 11, 13 and 14 (listed in Table 4).

Preferably, in the composition for use as described above, said vaginal infection is selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis due to at least a pathogen selected from the group comprising: *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis* and *Gardnerella vaginalis*.

Advantageously, the inventors found that the bacteria of the strain *Lactobacillus fermentum (*LF05) with deposit number DSM 32277 or CNCM I-789 (depositor Probiotical SpA) act against different species of yeasts of the genus *Candida*, as well as against bacteria, such as *Gardnerella vaginalis*, which are the main causes of bacterial vaginitis. In this respect, the bacteria of the strain *Lactobacillus fermentum (*LF05) with deposit number DSM 32277 or CNCM I-789 can be used for the treatment of disorders due to the concurrent presence of yeasts of the genus *Candida*, among which, inter alia, *Candida albicans*, and bacteria, among which, but not limited to, *Gardnerella vaginalis*.

Advantageously the composition of the present invention is for vaginal or oral use for the treatment of vaginal infections, disorders or diseases due to the association of the pathogens *Candida albicans* and *Gardnerella vaginalis*, in that *Lactobacillus fermentum (*LF05) was found to be active against yeasts, such as *Candida albicans* and, surprisingly, also against *Candida* species other than *albicans,* and the bacterium *Gardnerella vaginalis*.

Advantageously, the composition of the present invention is for vaginal or oral use for the treatment of vaginal infections, disorders or diseases selected from candidiasis, among which fungal candidiasis, vaginitis and bacterial vaginosis.

An embodiment of the present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement or a composition for a food product comprising pharmaceutical- and/or food-grade excipients and a mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum*; said composition being for vaginal or oral use for the treatment of at least a vaginal infection, wherein said at least a vaginal infection comprises or consists of bacterial vaginosis, caused by the pathogenic bacterium *Gardnerella vaginalis* and wherein the bacteria belong to the strain *Lactobacillus fermentum (*LF05) with deposit number DSM 32277 or CNCM I-789 (depositor Probiotical SpA).

Preferably, in the composition of the invention, said mixture further comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria from 3 to 14 below:

List of tested strains	Abbreviation	ID	Deposit	Depositor
No. 3 *L. fermentum*	LF 06	1456	DSM 18295	Anidral Srl
No. 4 *L. fermentum*	LF 07	1459	DSM 18296	Anidral Srl
No. 5 *L. fermentum*	LF 08	1460	DSM 18297	Anidral Srl
No. 6 *L. fermentum*	LF 09	1462	DSM 18298	Anidral Srl
No. 7 *L. fermentum*	LF 10	1637	DSM 19187	Anidral Srl
No. 8 *L. fermentum*	LF 11	1639	DSM 19188	Anidral Srl
No. 9 *L. fermentum*	DPPMA 114	1757	DSMZ 23757	Probiotical SpA
No. 10 *L. fermentum*	Lb2	1753	DSM 16143	Probiotical SpA
No. 11 *L. fermentum*	LF 15	1852	DSM 26955	Probiotical SpA
No. 12 *L. fermentum*	LF16	1853	DSM 26956	Probiotical SpA
No. 13 *L. fermentum*	LF18	1897	DSM 29197	Probiotical SpA
No. 14 *L. fermentum*	LF 25	1956	DSM 32275	Probiotical SpA

In a preferred embodiment, said mixture further comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 7, 8, 11, 12, 13 and 14.

In a preferred embodiment, said mixture further comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 8, 11, 13 and 14.

In a preferred embodiment, the composition according to the present invention is for use in the treatment of at least a vaginal infection comprising, in addition to a bacterial infection caused by the pathogenic bacterium *Gardnerella vaginalis,* a vaginal infection, vaginitis, vulvovaginitis or bacterial vaginosis due to at least a pathogen selected from the group comprising: *Candida albicans*, *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis, Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Herpes simplex* and *Hemophilus Ducreyi*.

In a preferred embodiment, the composition according to the present invention is for use in the treatment of at least a vaginal infection comprising, in addition to a bacterial infection caused by the pathogenic bacterium *Gardnerella vaginalis,* a vaginal infection due to the pathogen *Candida albicans*.

The composition of the present invention can be in a solid form or in a liquid form. As a solid form it can be in the form of powder, granules, tablet or lozenge, whereas as a liquid form it can be in the form of solution, vaginal douche, dispersion or gel.

Preferably, said mixture contains said strains of bacteria at a concentration comprised from $1x10^8$ to $1x10^{12}$ CFU/g, preferably from $1x10^9$ to $1x10^{11}$ CFU/g.

Preferably, said composition contains said strains of bacteria at a concentration comprised from $1x10^6$ to $1x10^{10}$ CFU/g, preferably from $1x10^7$ to $1x10^9$ CFU/g.

Preferably, said mixture of bacteria is in said composition in a weight ratio comprised from 1:2 to 1:10, preferably in a weight ratio comprised from 1:3 to 1:5.

The composition of the present invention is for vaginal use and oral use and is effectively applied for the treatments of vaginal infections, disorders or diseases, in that it has a wide-spectrum activity against pathogenic microorganisms such as the microorganism *Candida albicans*, a yeast responsible for candidiasis, vaginitis, vulvovaginitis and the bacterium *Gardnerella vaginalis*, which causes bacterial vaginosis. Said composition can also be useful for vaginal applications for the treatment of vaginal infections, disorders or diseases such as gonorrhea, herpes and chancroid.

The strains of bacteria listed in Table 4 can be in said bacterial mixture either as live or dead bacteria, or in the form of sonicated, tyndallized or freeze-dried bacteria.

In a preferred embodiment, the strains of bacteria belonging to the species *L. fermentum* being present in said bacterial mixture contained in said composition of the present invention are in a coated form with one (single coating) or more (for example double coating) plant materials for lipid coating having a melting point comprised from 30°C to 80°C, preferably from 40°C to 60°C.

The composition of the present invention comprises at least a strain of probiotic bacteria belonging to the species *L. fermentum* able to reducing and/or eliminating the presence of pathogenic agents selected from

the group comprising: *Candida albicans*, *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis, Gardnerella vaginalis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Herpes simplex* and *Hemophilus Ducreyi*.

As an example, in the tablets according to the invention, the strains of bacteria, preferably used in the form of freeze-dried culture having a viable count usually comprised from 10 to 200 billions of colony-forming units (CFU)/gram, are preferably in an amount from 0.5 to 20% w/w, preferably from 1 to 15% w/w, even more preferably from 3 to 10% w/w, relative to the overall weight of the tablet.

In one of the preferred embodiments, in order to enhance the efficacy of the formulations according to the present invention, specific prebiotic components are added to the powdery mixture thus obtaining a symbiotic composition. The prebiotic component is usually a not digestible saccharide material, at least partially soluble in water or aqueous solution, which stimulates the growth and/or activity of one or more strains of probiotic bacteria as described above. Among these prebiotic agents, food fibers are preferred.

Preferably, said prebiotic fiber is selected from the group comprising: fructooligosaccharides (FOS), galactooligosaccharides (GOS), trans-galactooligosaccharides (TOS), xylooligosaccharides (XOS), chitosan oligosaccharides (COS), α-galactosides (such as raffinose and stachyose), pectins, gums, partially hydrolyzed gums, inulin, psyllium, acacia, carob, oat, bamboo fiber, citrus fibers and, in general, fibers containing a soluble and an insoluble portions, with variable ratios each other.

Advantageously, said prebiotic fiber is selected from fructooligosaccharides (FOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS). These fibers are not exploited by yeasts of the genus *Candida*, thus providing a competitive advantage for the bacterial strains of the composition of the present invention.

Preferably, the prebiotic component is present in the composition in an amount up to 70% w/w, preferably comprised from 5 to 50% w/w, even more preferably from 10 to 30% w/w, relative to the overall weight of the composition.

In a preferred embodiment, the composition according to the invention can contain additional active components, for example vitamins, mineral salts, plant extracts or other compounds with synergistic or complementary effect to that of the microorganism population of the formulations according to the invention.

Preferably, said additional active components are in an amount up to 70 w/w, preferably comprised from 0.5 to 40% w/w, even more preferably from 1 to 20% w/w, relative to the overall weight of the composition.

In an embodiment (E1) the present invention relates to a pharmaceutical composition or a composition for a medical device or a composition for a food supplement or a composition for a food product comprising pharmaceutical- and/or food-grade excipients and a mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum*; said composition being for vaginal or oral use for the treatment of vaginal infections.

In a preferred embodiment (E2), the present invention relates to the composition for use according to embodiment E1, wherein said vaginal infections are selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis.

In a preferred embodiment (E3), the present invention relates to the composition for use according to embodiment E1 or E2, wherein said mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria from 1 to 14 below:

List of tested strains	Abbreviation	ID	Deposit	Depositor
No. 1 *L. fermentum*	LF 05	686	DSM 32277	Probiotical SpA
No. 2 *L. fermentum*	LF 05	686	CNCM I-789	Probiotical SpA
No. 3 *L. fermentum*	LF 06	1456	DSM 18295	Anidral Srl
No. 4 *L. fermentum*	LF 07	1459	DSM 18296	Anidral Srl
No. 5 *L. fermentum*	LF 08	1460	DSM 18297	Anidral Srl
No. 6 *L. fermentum*	LF 09	1462	DSM 18298	Anidral Srl
No. 7 *L. fermentum*	LF 10	1637	DSM 19187	Anidral Srl
No. 8 *L. fermentum*	LF 11	1639	DSM 19188	Anidral Srl
No. 9 *L. fermentum*	DPPMA 114	1757	DSMZ 23757	Probiotical SpA
No. 10 *L. fermentum*	Lb2	1753	DSM 16143	Probiotical SpA
No. 11 *L. fermentum*	LF 15	1852	DSM 26955	Probiotical SpA
No. 12 *L. fermentum*	LF16	1853	DSM 26956	Probiotical SpA
No. 13	LF18	1897	DSM 29197	Probiotical SpA

L. fermentum				
No. 14 *L. fermentum*	LF 25	1956	DSM 32275	Probiotical SpA

In a preferred embodiment (E4), the present invention relates to the composition for use according to embodiment E3, wherein said mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 1, 2, 7, 8, 11, 12, 13 and 14.

In a preferred embodiment (E5), the present invention relates to the composition for use according to embodiment E4, wherein said mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 1, 2, 8, 11, 13 and 14.

In a preferred embodiment (E6), the present invention relates to the composition for use according to any one of embodiments E1-E5, wherein said composition is for the treatment of vaginal infections, disorders or diseases due to the pathogens *Candida albicans* and *Gardnerella vaginalis*.

In a preferred embodiment (E7), the present invention relates to the composition for use according to any one of embodiments E1-E6, wherein said composition is in a solid form or liquid form, preferably in the form of powder, granules, tablet, lozenge, solution, vaginal douche, dispersion or gel.

In a preferred embodiment (E8), the present invention relates to the composition for use according to any one of embodiments E1-E7, wherein said mixture contains said strains of bacteria at a concentration comprised from $1x10^8$ to $1x10^{12}$ CFU/g, preferably from $1x10^9$ to $1x10^{11}$ CFU/g.

In a preferred embodiment (E9), the present invention relates to the composition for use according to any one of embodiments E1-E8, wherein said composition contains said strains of bacteria at a concentration comprised from $1x10^6$ to $1x10^{10}$ CFU/g, preferably from $1x10^7$ to $1x10^9$ CFU/g.

In a preferred embodiment (E10), the present invention relates to the composition for use according to any one of embodiments E1-E9, wherein said mixture of bacteria is present in said composition in a weight ratio comprised from 1:2 to 1:10, preferably in a weight ratio comprised from 1:3 to 1:5.

Experimental part

Biological anti-*Candida* spp. activity specific of the strain *L. fermentum* LF5 – "in vitro" study.

1. Selection of the optimal liquid and agar substrate

for the growth of microorganisms: *Candida albicans* and *L. fermentum*.

Growth tests for *C. albicans* ATCC 90028 and the strains *L. fermentum* LF5 (Table 5) were conducted on several liquid and agar substrates, under different conditions and for more or less long periods of

incubation. The growth was assessed based on the turbidity of the culture medium by reading the absorbance at 560 nm of the cultures themselves, see table 5.

		Candida albicans **ATCC 90028**	***L. fermentum*** **LF5**
MRS liquid medium 37°C			
aerobiosis	pH 6	**+++**	**+++**
	pH 5.5	**++(+)**	**+++**
	pH 5	++(+)	n.p.
	pH 4.5	++	n.p.
	pH 4	+	n.p.
Sabouraud liquid medium 37°C			
aerobiosis	pH 5.5	**+++**	**++(+)**
	pH 5	**+++**	++
	pH 4.5	**+++**	n.p.
	pH 4	**++(+)**	n.p.
Sabouraud agar medium 30°C			
aerobiosis (spread plating)		+++	-
aerobiosis (inclusion)		+++	-
anaerobiosis (inclusion)		n.p.	-
MRS agar medium 30°C			
aerobiosis (spread plating)		+++	++ (48h ϕ 1mm)
anaerobiosis (spread plating)		+/-	'- (24h)/+++ (48h Ø 2mm)
MRS agar medium 37°C			
aerobiosis (spread plating)		+++	+++ (24h Ø 1mm)
aerobiosis (spread plating)		-	+++ (24h Ø 2mm)

Table 5 Growth of *C. albicans* ATCC 90028 and *L. fermentum* LF5 on several substrates under various incubation conditions.

Therefore, the following conditions, as the perfect compromise for co-growing the strains *L. fermentum* and *C. albicans* ATCC 90028, were detected:

a) liquid or agar MRS as the growth substrate;

b) 37°C as the incubation temperature (preferential for lactobacillus);

c) under aerobiosis (a condition observed for a good growth of the yeast).

2. Assessment of the biological activity of the strain *L. fermentum* LF5 co-grown with C. albicans.

2.1 Assessment of the biological activity against *Candida albicans*

Growth tests in which *Candida albicans* ATCC 90028 was inoculated (10^4 cell/ml) in liquid medium inoculated with the strain *L. fermentum* (10^3 cell/ml) were established. The inhibition of the yeast induced by the bacterium was assessed by directly counting it in agar medium after 24 and 48 hours of co-growth.

(a) In view of the average of the values from the counts, the strain LF5 (Table 4) can be affirmed to determine an inhibition of *C. albicans* ATCC 90028 equal to 99.9% after 24 hours and 99.999% after 48 hours. Therefore, when the number ratio between inoculated cells of *C. albicans* and *L. fermentum* is 10 to 1 respectively, a $MCB_{99.9}$ after 24 hours of incubation and a $MCB_{99.999}$ after 48 hours of contact are obtained.

b) The specific inhibition, induced by *L. fermentum* LF5 against *C. albicans*, is not equally found in the co-growth of the yeast with other strains of lactobacillus. The number of *Candida* colonies being counted in these samples is in any case lesser than that obtained when growing the yeast alone in MRS. This phenomenon can be ascribed to the spontaneous acidification of the medium occurring during the growth of lactobacilli, as well as definitely to the competition for nutrients and the presence of various catabolic products from lactic acid bacteria. In the substantial inhibition observed for *C. albicans* by *L. fermentum* LF5 and overall quantified above, a specific activity of the strain LF5 takes place, which distinguishes it from other acidifying lactobacilli (Graph 1).

c) It is further demonstrated that *L. fermentum* LF5 maintains the capability of inhibiting the growth of *C. albicans* in a liquid medium even after several passages in a culture thereof starting from MCB (Master Cell Bank), up to even the eighth sub-culture, it was therefore possible to start the production of the strain LF5 in a pilot plant and then realize the scaling-up at an industrial level. It was then proven the maintenance of the specific activity against the yeast even in the final freeze-dried product.

2.2 Assessment of the biological activity against other strains belonging to the genus *Candida* spp.

According to the same operating modes as above, co-growth tests for the strain *L. fermentum* LF5 along with other strains of *Candida*: *Candida glabrata ATCC 90030*, *Candida Krusei ATCC 6258*, *Candida parapsilosis ATCC 22019* were performed.

The strain *L. fermentum* LF5 is able to inducing a substantial degree of mortality (Graphs 2, 3, 4), slightly varying according to the species, as well as the conditions under which it is assessed, of the above different strains of *Candida*. On average, an inhibition of about 99.9% during the first 24 hours of incubation is observed, with a mortality reaching 99.99 – 100% during the 48 hours depending on the

given strain. In view of the fact that the species *Candida parapsilosis* and *glabrata* are opportunistic pathogens, which can lead to both infections at the skin surface level, and systemic disorders it can be supposed an application of the strain LF5 as active ingredient for future pharmaceutical formulations, not for systemic use only with urogenital and intestinal efficacy, but also for topical use at the skin surface level.

2.3 Hypothesis for the possible mechanism of action of *L. fermentum* LF5 against *C. albicans* ATCC 90028

By microscope observation of the co-growth in LF5 and C. *albicans* liquid medium and digital image capture, it was possible to evaluate the action of the lactobacillus, which seems to perform a kind of yeast "opsonization", likely followed by lysis of the membrane through a specific, presumably protein factor bound to the membrane and possibly secreted (Figure 1).

3. Assessment of the biological activity of the supernatant of fermentation

Growth tests for *C. albicans* ATCC 90028, inoculated into the fermented broth from LF5 (supernatant) were performed. The supernatant represents the culture medium in which *L. fermentum* LF5 (10^6 cell/ml), under optimum conditions for the lactobacillus, was developed and then cell deprived through filtration (Table 6).

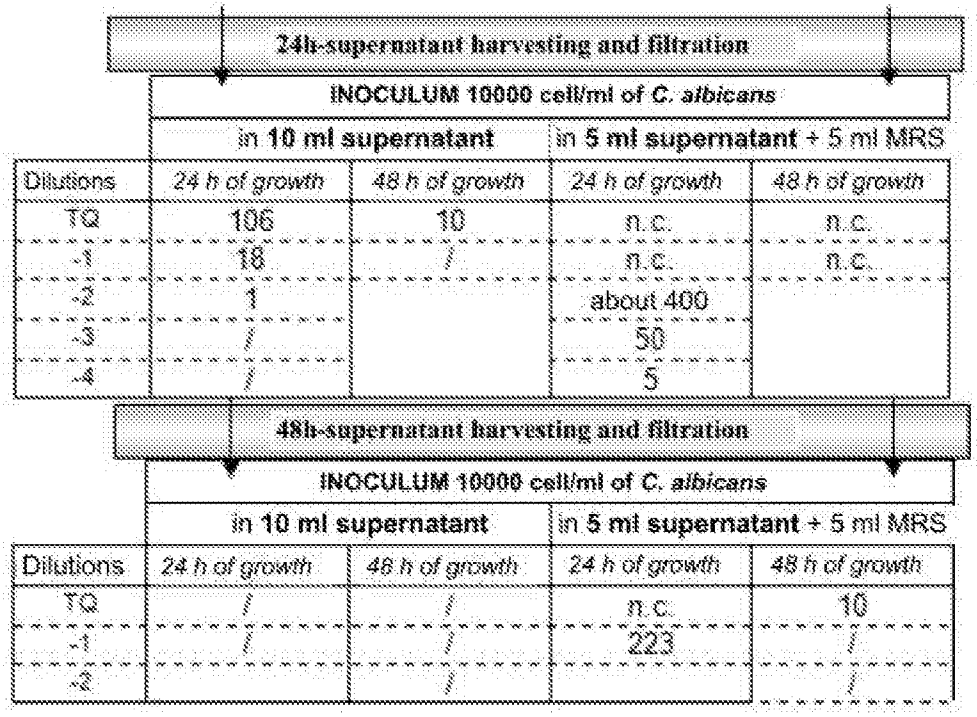


Table 6 C. albicans ATCC 90028 population after 24- and 48-hour growth in LF5 supernatant, with a positive control represented by the count of *Candida* inoculated into 10 ml MRS after 24 h ($5.7x10^6$ cell/ml).

a) The "activity" leading to the inhibition of *Candida*, being observed in the co-growth experiments (item 2.1) is maintained in the supernatant, presumably through a specific “factor” released from LF5 even in the culture broth.

b) Such an inhibition activity against the yeast is directly proportional to the concentration of supernatant into which it is inoculated and thus presumably to the concentration of the “factor” released by the lactobacillus in the culture broth (i.e. only supernatant and diluted 1:2 Table 6).

c) This concentration increases during the 48 hours of *L. fermentum* growth (i.e. No. of yeast colonies counted after 24 hours from inoculation, in the 24- and 48-hour supernatant).

d) Given the same concentration, the inhibition activity increases as contacting time increases (i.e. No. of colonies counted in the 24-hour supernatant after 24 and 48 hours of incubation).

e) Specifically, the inhibition induced by the broth fermented by LF5, after 24 hours of incubation and cell deprived through filtration, against *C. albicans* ATCC 90028 is 99.99% at 24 hours and 99.999% at 48 hours. Such an inhibition is due to the specific activity of LF5 as well as definitely to the acidity of the

growth substrate into which the yeast (supernatant) is inoculated, and to some nutrient deficiency. In any case, additional inhibition tests for *Candida*, inoculated into the LF5 supernatant, by adjusting the pH and adding glucose and peptone in advance, shown the presence therein of a specific activity, which determines by itself a yeast inhibition of about 96% and 99% at 24 and 48 hours, respectively.

f) The inhibition efficiency of the LF5 supernatant increases when it is sub-coltured in MRS at temperatures greater than 37°C; in particular 42°C results to be the optimal condition for producing a higher concentration of the specific anti-*Candida* "factor". Conversely, the growth of LF5 at controlled pH of 5 or 5.5 seems to not affect such a concentration/productivity.

The growth of *C. albicans* ATCC 90028 in agar medium, seeded by spread plating (10^4 cells), contacted with the broth culture and LF5 supernatant, deposited on agar by the spot or impregnated disc technique was assessed.

a) In the plates with the disc (Figure 2) in correspondence of that impregnated with broth culture of LF5 TQ, an inhibition of the *Candida* growth around the disc occurs, with the appearance of a well-detectable halo of about 5 mm from the disc edge.

b) The technique of the impregnated disc, put on agar medium previously spread plated with the yeast, generally leads to a more naked-eye detectable inhibition halo and/or lysis by L. *fermentum*, relative to the spot technique: in the plates with spots, however, within the drop of LF5 TQ, single cells of yeast with not well-defined profiles can be microscopically observed, as if cell debris is present and the area among the cells is dark and inhomogeneous. The condition outside the drop is different, where the yeast cells have well-defined profiles and the agar bottom is clearer and almost homogeneous. What is detectable inside the drop can be ascribed to an inhibition of the yeast growth, with the presence of possible debris due to the lysis, along with lactobacillus cells which grown anyway.

c) The agar tests carried out with discs and/or spots of the supernatant, harvested from a LF5 culture broth, determined no formation of detectable halos probably because of the difficulty for the "factor" with specific activity to spreading into the agar. The freeze-dried and reconstituted supernatant, in order to concentrate, even up to 20 times, such a factor, leads to the occurrence of clear inhibition halos (Figure 3) with both the disc and spot techniques as well as by directly using the not reconstituted powder (Figure 4-5-6).

4. Clinical study for the efficacy and tolerability of LF5 vaginal capsules compared with placebo in patients with *Candida albicans*

100 patients with vaginitis or vulvovaginitis due to *Candida albicans*, average age of 32.7 years (range 18-64 years) randomly assigned to two groups, which resulted homogeneous, each of 50 patients, treated

with placebo o LF5 dosed at ≤10^9 in a vaginal capsule formulation at a dosage of 1 vaginal capsule at night for three consecutive days, according to the experimental double-blind design were treated.

No discontinuations during the treatment nor during the two weeks of post-treatment observation occurred, thus two groups of 50 observations for efficacy and tolerability are provided.

The treatment with LF5 produced microbiological eradication at the end of the three-day treatment in a proportion of patients significantly greater than placebo, along with a very low risk of relapses during the next two weeks. Furthermore, even the symptom remission was significantly greater with LF5 compared with placebo.

The efficacy data relative to the comparison LF5 vs placebo are reported below in Table 7a and figure 7.

Table 7a Results of the study for LF5 efficacy compared with placebo

Parameter		Placebo	LAB (LF5)	Statistics
Positive/negative	Baseline	50/0	50/0	-
	3 Days	14/36	2/48	0.002**[1]
	Final	40/10	5/45	<0.001***[1]
	Chi square	164.967	122.750***	
Eradication	Failure	14	2	44.673***[2]
	Relapse	26	3	
	Success	10	45	

**P<0.01; ***0.001

[1]Fisher

[2]Mann-Whitney

Table 7b: Patient's opinion about efficacy

Opinion	Placebo	LAB (LF5)	Mann-Whitney[1]
Excellent	4	42	53.322**
Good	6	3	
Acceptable	26	3	
None	14	2	

***0.001

[1]Chi-square approximation

4.1 Tolerability study

The clinical tolerability was good with both the preparations, with 3 cases of poor local reactions with LF5 (6%) and 1 with placebo (2%), with a not statistically relevant difference between the two groups. No clinically relevant changes for peripheral hemodynamics or routine hematology were observed.

The tolerability data relative to the comparison LF5 vs placebo are reported below in Table 7b and figure 7.

Table 7c Results of the study for LF5 tolerability compared with placebo

Opinion	Placebo	LAB (LF5)	Mann-Whitney[1]
Tolerability			-
Excellent	49	47	0.002**[1]
Good	3	1	1.031
Acceptable	0	0	
None	0	0	
Adverse effects			0.617[2]
Present	1	3	
Adverse	49	47	

[1] Chi-square approximation

[2] Fisher's exact test

The strain LF5 has an efficacy and tolerability profile in the treatment of vaginal colonization by *Candida albicans* which is definitely favorable and, in any case, significantly more favorable relative to placebo, because of a statistically higher microbiological and clinical efficacy, with the same risk of poor, not clinically relevant local reactions. LF5 in vaginal capsules is thus suggested as a valid alternative to synthetic antifungal drugs for the treatment of vaginal candidiasis.

Examples of tested compositions

Composition of LF5 proprietary medicinal products

Each vaginal capsule contains:

Active ingredient:

Freeze-dried *Lactobacillus Fermentum* No. 1 and 2 (Table 4) NLT 10^9 CFU

Excipients:

Medium-chain triglycerides 1.964 mg

Silica (Aerosil 300) 36 mg

Shell composition:

Gelatin F.U.	400 mg
Glycerol F.U.	200 mg
Dimethylpolysiloxane 1000	90 mg
Titanium dioxide E 171	9 mg

Each capsule of dermatological oily suspension contains: Active ingredient:	
Freeze-dried *Lactobacillus Fermentum* No. 1 and 2 (Table 4)	NLT 10^9 CFU
Excipients:	
Medium-chain triglycerides	1.964 mg
Silica (Aerosil 300)	36 mg
Shell composition:	
Gelatin F. U.	400 mg
Glycerol F.U.	200 mg
Titanium dioxide E 171	9 mg
Dimethylpolysiloxane 1000	90 mg

5 Clinical study for the efficacy and tolerability of LF5 vaginal capsules compared with miconazole in patients with *Candida albicans*

100 patients with vaginal candidiasis, average age of 38.0 years (range 19-61 years) randomly assigned to two groups, which resulted homogeneous, each of 50 patients treated with miconazole or LF5 dosed at ≤ 10^9 in vaginal capsule formulation at a dosage of 1 vaginal capsule at night for three consecutive days, according to the single-blind experimental design were treated.

No discontinuations during the treatment nor during the two weeks of post-treatment observation occurred; thus two groups of 50 observations for efficacy and tolerability are provided.

Both the treatments produced, at the end of the three-day treatment, microbiological eradication of Candida in almost all the patients (96% vs. 94%). The risk of relapse during the two weeks following to the treatment also resulted very low with both the treatments. However, the relapse risk with miconazole (8/47 patients; 17%) was appreciably higher relative to LF5 (5/48 cases; 10%). The symptom remission was very favorable with both the treatments as well.

The clinical tolerability was good with both the preparations. The frequency of local adverse events, however, was three-fold greater with miconazole (6 cases; 12%) relative to LF5 (2 cases; 4%). No clinically relevant changes for peripheral hemodynamics or routine hematology were observed.

The efficacy data relative to the comparison LF5 vs miconazole are reported below in Table 8a and figure 8a and 8b.

Table 8a Results of the study for the LF5 efficacy compared with placebo

Parameter		LAB (LF5)	Miconazole	Statistics
Positive/negative	Baseline	50/0	50/0	-
	3 days	2/48	3/47	1.000[1]
	Final	5/45	8/42	0.554[1]
	Chi square	122.750	110.462***	
Eradication	Failure	2	3	0.774***[2]
	Relapse	3	5	
	Success	45	42	

***0.001

[1]Fisher

[2]Mann-Whitney

Table 8b: Patient's opinion about efficacy

Opinion	LAB (LF5)	Miconazole	Mann-Whitney[1]
Excellent	40	38	53.322***
Good	5	4	
Acceptable	3	5	
None	1	3	

***0.001

[1]Chi-square approximation

The tolerability data relative to the comparison LF5 vs miconazole are reported below in Table 8c.

Table 8c Results of the study for LF5 tolerability compared with miconazole

Opinion	LAB (LF5)	Miconazole	Mann-Whitney[1]
Tolerability			-
Excellent	48	44	2.148
Good	1	3	
Acceptable	1	3	
None	0	0	
Adverse events			
Present	1	3	0.269[2]
Adverse	49	47	

[1]Chi-square approximation

[2]Fisher's exact test

In conclusion, LF5 presents an efficacy and tolerability profile in the treatment of vaginal candidiasis, which is definitely favorable and substantially equal, as regards the profile of microbiological and clinical efficacy, to a well-established drug such as miconazole. However, the LF5 tolerability, even without reaching a statistical significance, was highly greater relative to that of the reference drug. Accordingly, LF5 vaginal capsules result a valid alternative to synthetic antifungal drugs for the treatment of vaginal candidiasis.

6 LF5: study for local tolerability and activity in patients with *Candida albicans*

100 patients with vaginitis, vulvovaginitis o more extensive *Candida albicans* colonization, average age of 34.0 years (range 17-65 years) with LAB dosed at $\leq 10^9$ in vaginal capsule formulation at a dosage of 1 vaginal capsule at night for 14 consecutive days, according to the open experimental design were treated.
No treatment discontinuations occurred, therefore 100 observations for efficacy and tolerability are provided.
The treatment with LF5 produced a microbiological eradication at the end of the first three days of treatment in 92% of patients, and in an additional 5% during the following period of treatment. Overall, only 3% of patients failed the therapy.
During the same observation period, a remarkable remission of the symptoms, in particular during the first three days of treatment was detected as well as, being statistically significant, during the remaining period of observation.
The clinical tolerability was good, with 5 total reports of poor local reactions. No clinically relevant changes in routine hematology were observed.
In conclusion, LF5 has an efficacy and tolerability profile in the treatment of vaginal colonization by *Candida albicans* which is definitely favorable, in that it already has a statistically significant microbiological and clinical efficacy during the first three days of application, with a further improvement when the therapy is continued, without relevant risks of local, always slight, clinically irrelevant and spontaneously reversible adverse reactions, nor detectable risks of alterations of the lab parameters.
Therefore, LF5 vaginal capsules are suggested as a valid alternative to synthetic antifungal drugs for the treatment of vaginal candidiasis, even when it spreads to the proximal structures and in patients with particular conditions such as pregnancy.

Methods

The aim of the method is to verify if a given bacterium is able to inhibit the broth growing of *Gardnerella vaginalis* by the production of substances with bacteriostatic/bactericidal action.

Materials and reagents:

- MRS Broth (Difco)
- Cysteine chlorohydrate sol. 5% aqueous solution, in sterile pure water. Sterilize through syringe filters 0.22 µm of diameter
- THB (Todd Hewitt Broth Sigma – Aldrich)
- Peptone saline solution (MET_INT 119-version in force)
- NaOH 30%
- *Gardnerella vaginalis* ATCC 14018 (Biogenetics)

Sampling and preparation of the sample:

Preparation of the inhibitory strain

Two sequential inoculations of the probiotic bacterium into 15 ml MRS broth (5.1) 0.1% and incubated at 37±1°C overnight are performed. When the bacterium belongs to the genus *Bifidobacterium* the culture broth is supplemented with 1% cysteine HCl (5.2).

A third inoculation with the same modes, except for a broth amount of 100 ml is carried out.

The incubation is continued up to 30±3 hours.

The broth culture is thus centrifuged and the supernatant separated from cells is brought to pH 6.6±0.2 by adding NaOH conc.

The supernatant is then filtered through 0.2 µm filters and stored at a temperature of 4-5°C.

Preparation of *G. vaginalis*

A pellet of *G. vaginalis* ATCC 14018 is inoculated into 10 ml THB medium supplemented with 1% Cysteine HCl (5.2) and incubated at 37±1°C overnight.

A sequential inoculation at 1% 37±1°C overnight is performed.

The fresh broth culture thus prepared is used for the inhibition experiment

Procedure:

The sterile tubes being prepared contain:

THB	10ml	9.5ml	9ml	8ml	6ml
MRS	0ml	0.5ml	1ml	2ml	4ml

Bacterial supernatant (7.5)		0ml	0.5ml	1ml	2ml	4ml

All the tubes thus prepared are added with 1% Cysteine HCl (5.2).

They are then inoculated at 1% with the fresh broth culture of *G. vaginalis* and incubated at 37±1°C for 24 + 24 hours.

At 24 and 48 hours spectrophotometric readings (wavelength λ600) by using for each set a blank with the same percentage composition as the two culture broths are conducted.

7. Comparative study for the efficacy relative to azole therapies

In view of the above, a combination of *Lactobacillus fermentum* strains was selected in vitro based on the ability thereof to fight different species of Candida and then compared with miconazole and fluconazole as regards the inhibition efficacy. Specifically, the possible use of Lactobacillus strains as microorganisms for the prophylaxis and/or adjuvant therapy of acute vulvovaginal candidiasis (VVC) and other vaginal infections due to Candida yeasts was studied.

Materials and methods

Strains and conditions of bacterial growth.

The strains of Candida being used in the present study were purchased from the American Type Culture Collection (ATCC). Specifically, the following biotypes were used: *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. glabrata* ATCC 2001 and *C. tropicalis* ATCC 750 (14).

The strains of lactobacilli of this study, all isolated from vaginal swabs of healthy female subjects or directly brushing the intestinal mucosa of healthy humans, were classified based on their phenotypic and genotypic characteristics: *L. fermentum* LF5 (DSM 32277), *L. fermentum* LF09 (DSM 18298), *L. fermentum* LF10 (DSM 19187) and *L. fermentum* LF11 (DSM 19188).

The probiotic strains were cultured overnight in De Man, Rogosa and Sharpe (MRS) medium (Difco, BD, Maryland). In all the experiments, fresh culture media for all the strains of Candida were used. Before the experiment, the single strains of *Candida* were cultured in Sabouraud Dextrose Broth (Difco, BD, Maryland), an optimal medium for yeasts, for 48 hours under aerobic conditions.

Every probiotic was then co-cultured in the same broth (MRS) with any one of said *Candida* microorganisms. The ratio between the inoculum of probiotic and yeast was 1:100 in favor of the latter. Incubation was carried out under aerobiosis at 37°C for 24 hours. Each culture (1 mL) was sampled after 24 hours of incubation for the selective enumeration of the yeasts, conducted on yeast extract glucose chloramphenicol (YGC) agar (Sigma-Aldrich, Milan, Italy). The YGC agar plates were incubated under

aerobic conditions at 37°C for 4 days in order to allow the growth of any present yeast. The colonies were then counted and the results expressed as the number of colony forming units (CFU) / mL.

Evaluation of the fungicidal activity. The minimum inhibitory concentration (MIC) for fluconazole was determined by E-test (bioMérieux Italy, Florence) with MHE agar. The plates were incubated under aerobic conditions at 30°C and read after 24 hours. Furthermore, since there is no specific E-test for miconazole, the MIC was determined by using the broth macrodilution method. We assessed the activity of serially diluted fluconazole and miconazole (Sigma-Aldrich, Missouri, USA) (from 1 mg / ml to 1 mg / ml) against the *Candida* strains. Serial dilutions of azoles were prepared in Sabouraud Dextrose Broth in the presence of strains of *Candida* and broths were incubated under aerobic conditions at 30°C and then read at an optical density (DO) of 560 nm. The MIC was defined as the lowest concentration of antibiotic, which completely inhibited the visible growth.

Results

Inhibitory activity of azoles against Candida strains. As it can be inferred from tables 8-9, a marked resistance for all the species of *Candida* was detected, except for *C. parapsilosis* ATCC 22019. As regards this biotype, a MIC of 1 mg/ml was obtained, thus confirming the slight sensitivity to fluconazole and miconazole.

Table 9 Assessment of the inhibitory activity of miconazole by using a macrodilution test of the culture medium (optical density, OD)

Miconazole (µg/ml)	***C. parapsilosis* ATCC 22019**	***C. albicans* ATCC 10231**	***C. tropicalis* ATCC 750**	***C. krusei* ATCC 6258**	***C. glabrata* ATCC 2001**
0	1.610	1.912	1.972	1.987	2.246
1	1.515	1.906	1.950	1.990	2.260
2.50	1.115	1.910	1.927	1.900	2.260
5.00	0.975	1.901	1.908	1.893	2.246
50	0.894	1.905	1.846	1.994	2.239
250	0.701	1.916	1.942	2.004	2.212
300	0.415	1.889	1.937	1.999	2.215
500	0.314	1.863	1.903	1.988	2.213
1.000	0.295	1.464	1.931	1.972	2.198

Table 10

MIC (minimum inhibitory concentration) of fluconazole from E_test (range 0.016-256 µg/ml). *p≤0.05

***Candida* species**	**MIC (µg/ml)**

Candida parapsilosis ATCC 22019	1*
Candida albicans ATCC 10231	>256
Candida tropicalis ATCC 750	>256
Candida krusei ATCC 6258	>256
Candida glabrata ATCC 2001	>256

Antagonistic effects of *L. fermentum* against *Candida*.

Once the resistance of most of the *Candida* species to the two reference drugs is detected, the ability of *Lactobacillus fermentum* to inhibit the yeast growth was assessed.

As shown in Fig. 9, all the tested strains of *Lactobacillus*, namely LF5, LF09, LF10 and LF11, shown the capability to significantly inhibit the growth of the five species of *Candida* by at least four logarithms. Furthermore, the best result obtained with miconazole against *C. parapsilosis* is even two logarithms lower. The results for LF5 are reported in Figure 10.

Interestingly, the dose of antibiotic in humans generally provides the management of a maximum of 400 mg/die: such a *per os* concentration peaks an average plasma concentration of 30-40 pg / ml, a value which is much lower than the efficacy cut-off in the case of resistant strains.

Discussion

The possible role of *L. fermentum* compared with miconazole and fluconazole against different species of Candida was tested in vitro.

Specifically, the growth capability of *Candida* species in the presence of increasing concentrations of miconazole and fluconazole by two distinct tests, the broth macrodilution test for miconazole and E-test for fluconazole, was quantified.

The present results are highly surprising and represent the first proof for an in vitro relatively poor efficacy of azoles, which are widely used in counteracting the growth of *Candida* species and for a high effectiveness of lactic acid bacteria belonging to the species *Lactobacillus fermentum*. The only inhibition test was detected with *C. parapsilosis* ATCC 22019, even though the overall results are little relevant. The poor efficacy of azoles could account for the frequent relapses of *Candida* infections.

Actually, a complete eradication of the yeast from the vaginal environment could be difficult to obtain, thus maintaining the conditions for a subsequent new development and clinical manifestation of unfavorable symptoms.

On the other side, the selected strains of *L. fermentum* according to the invention confirmed the possibility to not only effectively and directly counteract the growth, but also the viability of several strains of Candida. One of these strains of *L. fermentum*, namely the strain LF5 (identified with deposit number

CNCM I-789) was used in women with acute VVC, but without any correlation with a specific species of *Candida* and not in the simultaneous presence of bacterial infection due to *Gardnerella vaginalis,* thus confirming the capability thereof in resolving the symptoms and rapidly eradicating the infection (Vicariotto F. et al. "*Effectiveness of the association of 2 probiotic strains formulated in a slow release vaginal product, in women affected by vulvovaginal candidiasis: a pilot study*". *J Clin Gastroenterol.* **2012**;*46* Suppl:S73-80).

8. Inhibition of *Gardnerella vaginalis* by *L. fermentum* LF5

This study was conducted in order to assess the ability of *L. fermentum* LF5 to directly inhibit *Gardnerella vaginalis* in vitro, so that to evaluate the potential efficacy thereof even in the event of mixed vaginal infections or BV.

Materials and methods

Bacterial strains and growth conditions.

The strain *Gardnerella vaginalis* being used was purchased from the American Type Culture Collection (ATCC). Before the experiment, *G. vaginalis* ATCC 14018 was cultured in a heart-brain infusion (BHI) broth (Oxoid, Milan, Italy) consisting of 2% gelatin (weight / weight), 0.5% yeast extract, 0.1% starch, and 0.1% glucose (7).

The strain *Lactobacillus fermentum* LF5 of this study was isolated from the vaginal swab of a healthy female subject and classified according to the phenotypic and genotypic characteristics thereof.

It was cultured overnight in De Man, Rogosa and Sharpe (MRS) broth (Difco, BD, Maryland).

Assessment of the antagonistic activity of LF5 against *G. vaginalis*.

LF5 supernatants were prepared as follows: an overnight culture in MRS broth was centrifuged at 5,000 x g. The resulting supernatant was neutralized at pH 6.5 with NaOH 1 N, sterilized by filtration through syringe filters (Ministart pore size 0.22 mm), and analyzed for the presence of any inhibitory molecule in the broth. Neutralized LF5 supernatants were then added with different percentages of fresh BHI broth, prepared as described above, inoculated with *G. vaginalis*. The growth of *Gardnerella* alone (positive control) and in the presence of different concentrations of neutralized supernatants ranging from 5% to 20%, after 24 and 48 hours of incubation at 37°C under microaerophilic conditions, was quantified by optical density at 600 nm (OD600). The growth of the positive control (Gardnerella alone) was assessed in BHI broth added to an amount of fresh MRS broth ranging from 5% to 20%. Thus, the positive controls were as similar as possible to the tubes inoculated with LF5. This test was repeated three times in order to ensure reliability and reproducibility.

Results

The in vitro inhibition results of LF5 against *G. vaginalis* ATCC 10231 are reported in Table 11. *L. fermentum* LF5 shown a significant activity after both 24 and 48 hours (46% and 82% with 20% neutralized supernatant, respectively). A significant inhibition of the dose-dependent growth was detected with the neutralized supernatants in particular after 48 hours of incubation, up to a growth inhibition of *G. vaginalis* of even 80% (Tab. 9).

Table 11

L. fermentum LF5 - biological activity against *G. vaginalis*.

LF5 supernatant (0.5, 1 and 2 mL) is able to inhibit the growth of *G. vaginalis* even after 24 hours, but the best inhibition is after 48 hours. Positive control = *G. vaginalis* - growth in an optimal medium plus an equal volume of MRS (5%, 10% and 20%) (n = 3).

Sample	**24h incubation**			**48h incubation**		
	OD = 600 nm					
	+5%	+10%	+20%	+5%	+10%	+20%
G. vaginalis + MRS (%)	1.379 ± 0.01	1.542 ± 0.02	1.557 ± 0.015	1.384 ± 0.014	1.554 ± 0.01	1.562 ± 0.016
G. vaginalis + LF5 (%)	**0.764 ± 0.008**	**0.645 ± 0.006**	**0.843 ± 0.007**	**0.562 ± 0.006**	**0.336 ± 0.004**	**0.275 ± 0.003**

The study shows that *L. fermentum* LF5 is able to exert a fundamental inhibitory activity against *G. vaginalis*, thus revealing a multipurpose application. The above-cited data show that the strain LF5 is also useful for the treatment of vulvovaginal candidiasis (VVC), due to different strains, among which, inter alia, *Candida albicans*; accordingly it can be used even in the clinical management of mixed vaginitis.

CLAIMS

1. Pharmaceutical composition or composition for a medical device or composition for a food supplement or composition for a food product comprising pharmaceutical- and/or food-grade excipients and a mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum*; said composition being for vaginal or oral use for the treatment of at least a vaginal infection, wherein said at least a vaginal infection is selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis, caused by at least a pathogen selected from the group comprising: *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis, Gardnerella vaginalis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Herpes simplex* and *Hemophilus Ducreyi*e and wherein the bacteria belong to the strain *Lactobacillus fermentum* (LF05) with deposit number DSM 32277 or CNCM I-789 (depositor Probiotical SpA).

2. The composition for use according to claim 1, wherein said mixture further comprises or, alternatively, consists of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria from 3 to 14 below:

List of tested strains	Abbreviation	ID	Deposit	Depositor
No. 3 *L. fermentum*	LF 06	1456	DSM 18295	Anidral Srl
No. 4 *L. fermentum*	LF 07	1459	DSM 18296	Anidral Srl
No. 5 *L. fermentum*	LF 08	1460	DSM 18297	Anidral Srl
No. 6 *L. fermentum*	LF 09	1462	DSM 18298	Anidral Srl
No. 7 *L. fermentum*	LF 10	1637	DSM 19187	Anidral Srl
No. 8 *L. fermentum*	LF 11	1639	DSM 19188	Anidral Srl
No. 9 *L. fermentum*	DPPMA 114	1757	DSMZ 23757	Probiotical SpA
No. 10 *L. fermentum*	Lb2	1753	DSM 16143	Probiotical SpA
No. 11	LF 15	1852	DSM 26955	Probiotical SpA

L. fermentum				
No. 12 *L. fermentum*	LF16	1853	DSM 26956	Probiotical SpA
No. 13 *L. fermentum*	LF18	1897	DSM 29197	Probiotical SpA
No.14 *L. fermentum*	LF 25	1956	DSM 32275	Probiotical SpA

3. The composition for use according to claim 2, wherein said mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 7, 8, 11, 12, 13 and 14.

4. The composition for use according to claim 3, wherein said mixture comprising or, alternatively, consisting of at least a strain of bacteria belonging to the species *Lactobacillus fermentum* selected from the group comprising or, alternatively, consisting of the strains of bacteria No. 8, 11, 13 and 14.

5. The composition for use according to at least any one of the preceding claims, wherein said vaginal infection is selected from candidiasis, vaginitis, vulvovaginitis or bacterial vaginosis due to at least a pathogen selected from the group comprising: *Candida glabrata, Candida parapsilosis, Candida krusei, Candida tropicalis* and *Gardnerella vaginalis*.

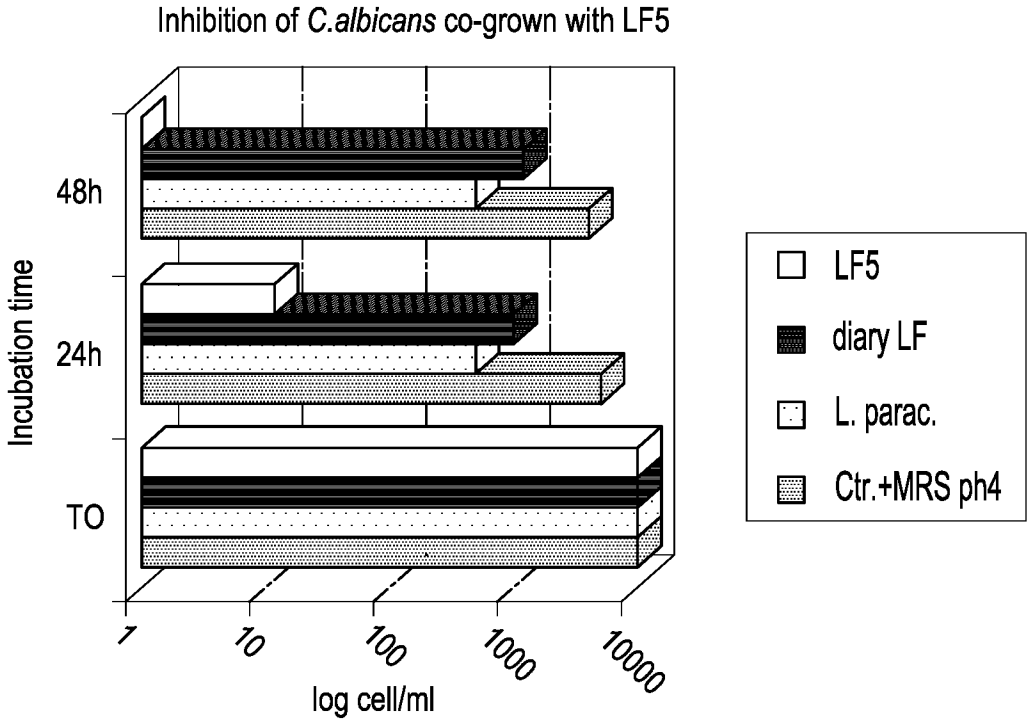
6. The composition for use according to any one of the preceding claims, wherein said composition is for the treatment of vaginal infections, disorders or diseases due to the pathogens *Candida albicans* and *Gardnerella vaginalis*.

7. The composition for use according to any one of the preceding claims, wherein said composition is in a solid form or liquid form, preferably in the form of powder, granules, tablet, lozenge, solution, vaginal douche, dispersion or gel.

8. The composition for use according to any one of the preceding claims, wherein said mixture contains said strains of bacteria at a concentration comprised from $1x10^8$ to $1x10^{12}$ CFU/g, preferably from $1x10^9$ to $1x10^{11}$ CFU/g.

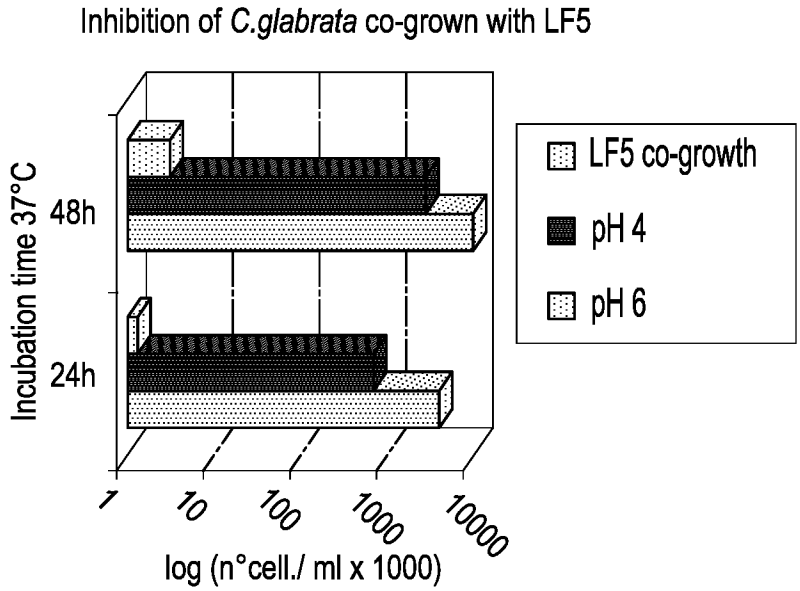
9. The composition for use according to any one of the preceding claims, wherein said composition contains said strains of bacteria at a concentration comprised from $1x10^6$ to $1x10^{10}$ CFU/g, preferably from $1x10^7$ to $1x10^9$ CFU/g.

10. The composition for use according to any one of the preceding claims, wherein said mixture of bacteria is present in said composition in a weight ratio comprised from 1:2 to 1:10, preferably in a weight ratio comprised from 1:3 to 1:5.



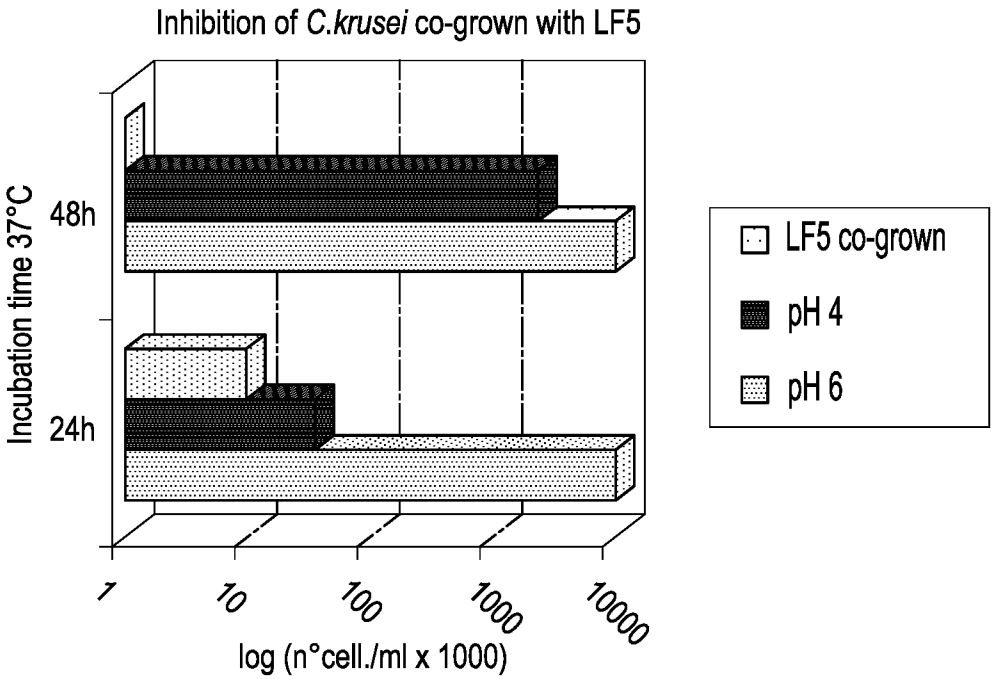
Illustrative histogram of the effect of the co-growth of lactobacilli and *C.albicans* in MRS

Graph 1



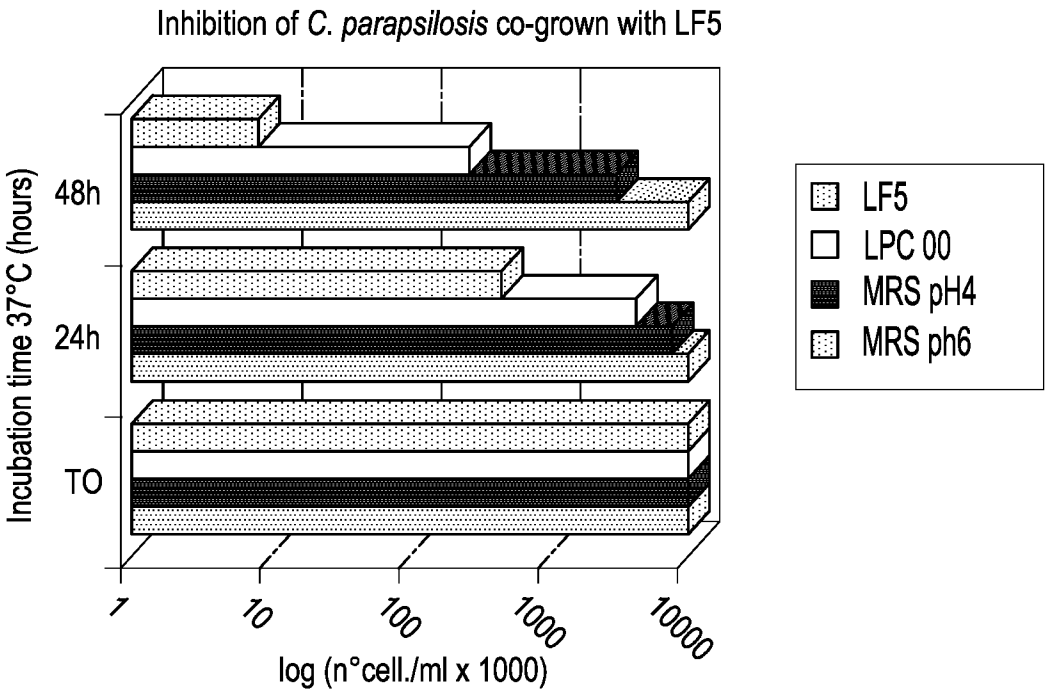
Histograms representing the mortality of the different species of Candida co-grown with LF5

Graph 2



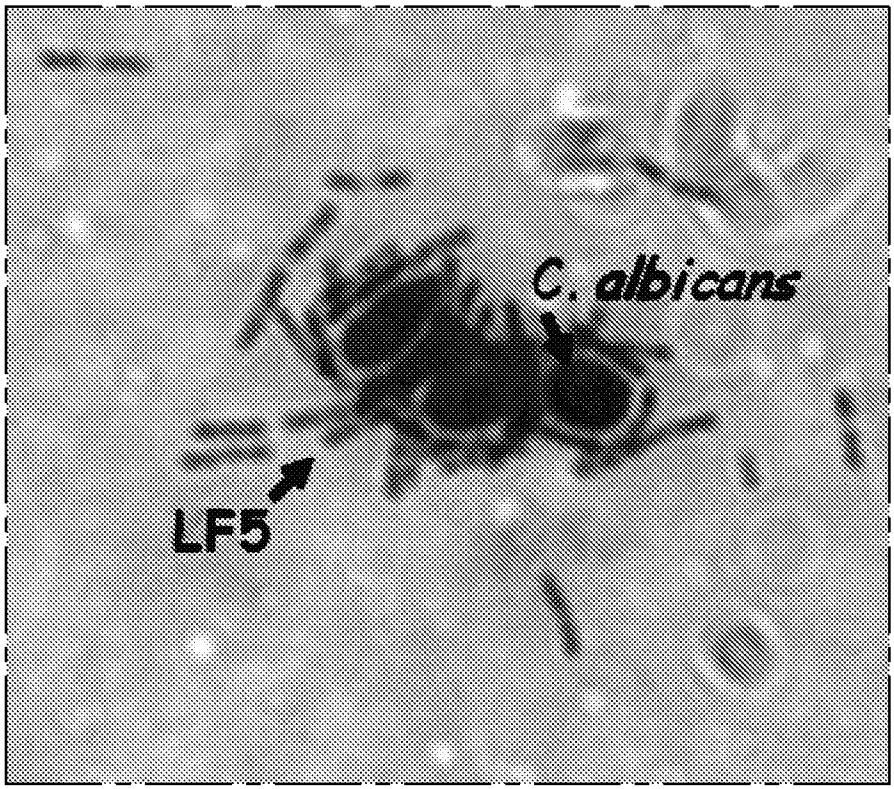
Histograms representing the mortality of the different species of Candida co-grown with LF5

Graph 3



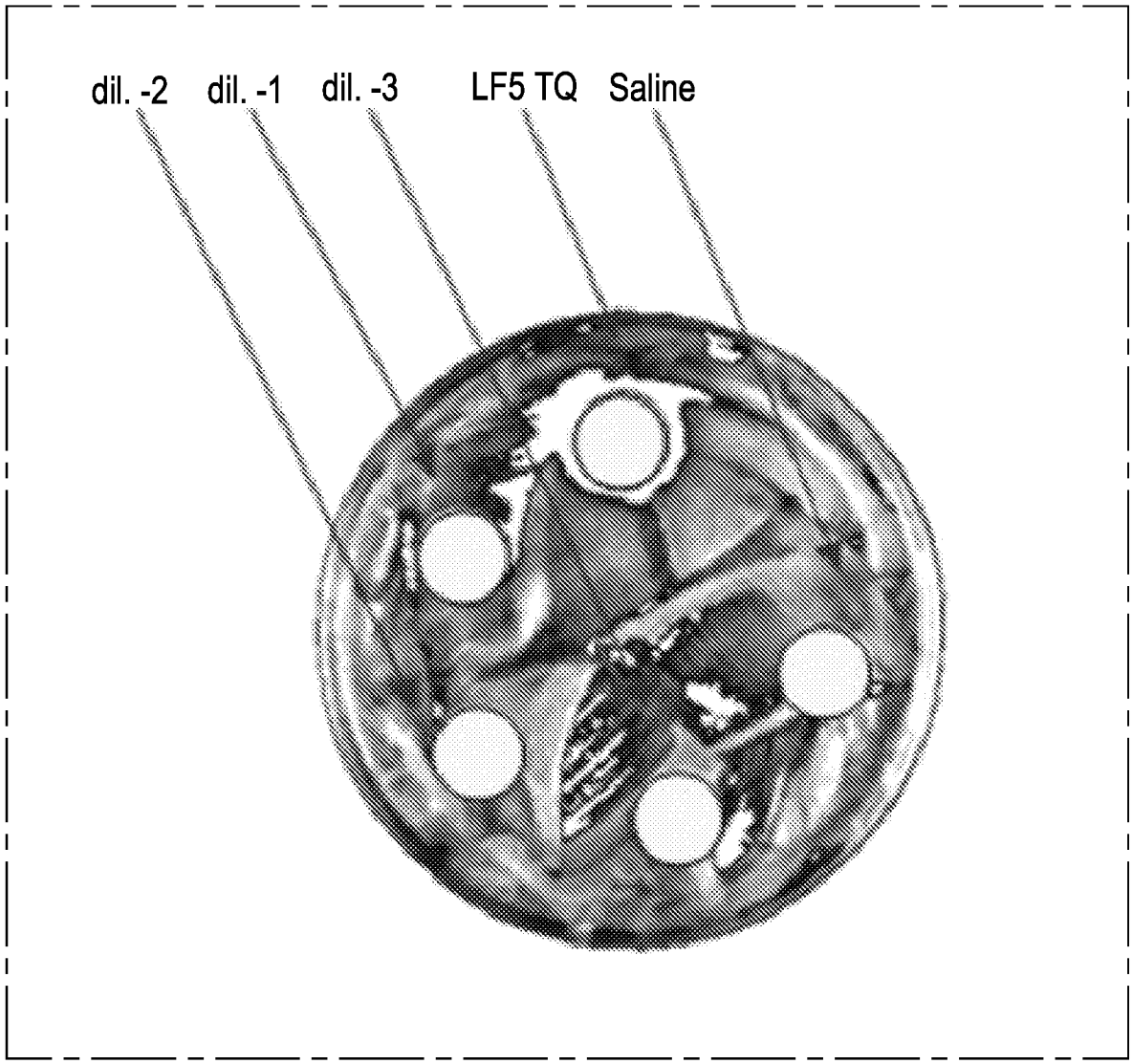
Histograms representing the mortality of the different species of Candida co- grown with LF5

Graph 4



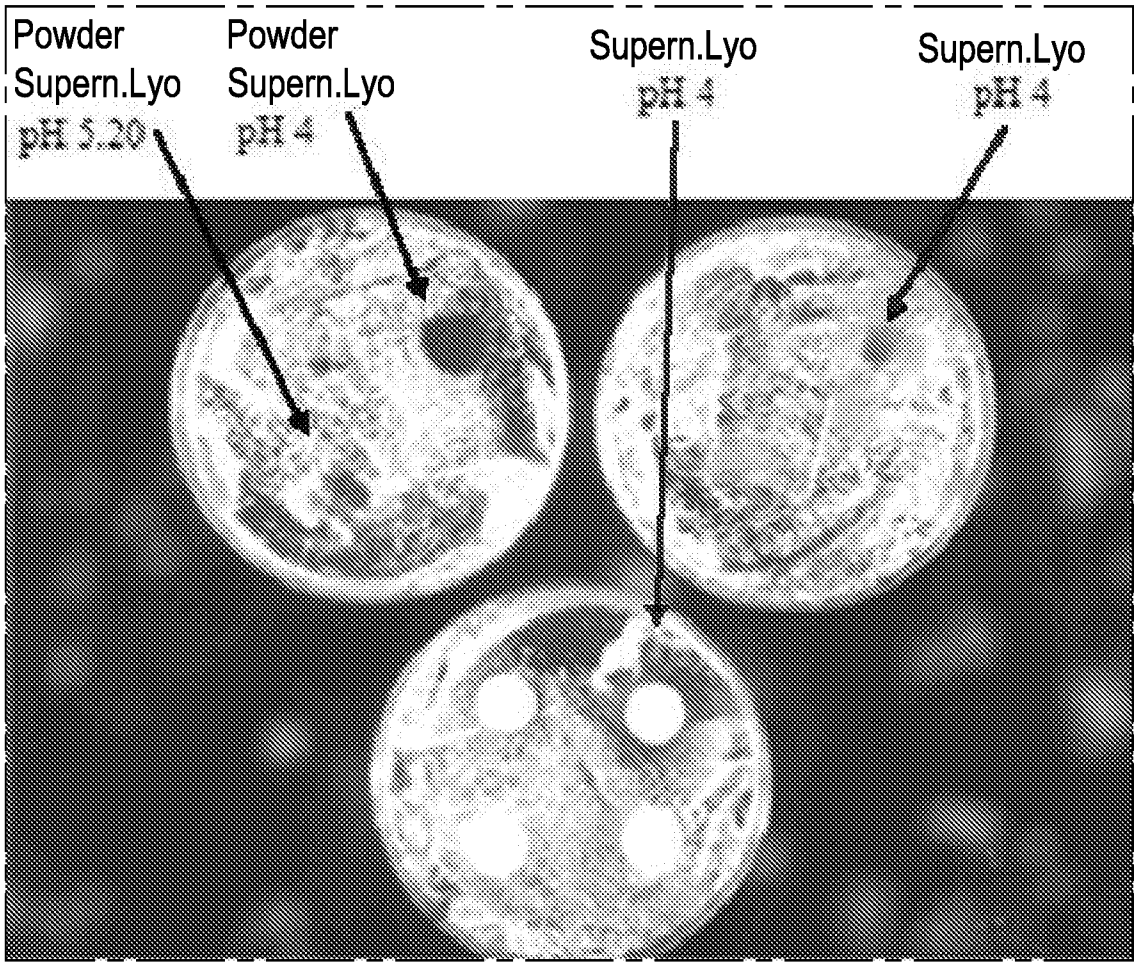
Action exerted by LF5 on C. albicans ATCC 90028 during the co-growth of the two microorganisms

FIG.1



Inhibition halos of *C.albicans* ATCC 90028 on agar medium induced by a broth culture of the strain LF5: disc technique

FIG.2



Inhibition halos of the growth of *C.albicans* ATCC 90028 on agar medium induced by the freeze-dried concentrated of the supernatant of LF5: disc and spot techniques

FIG.3

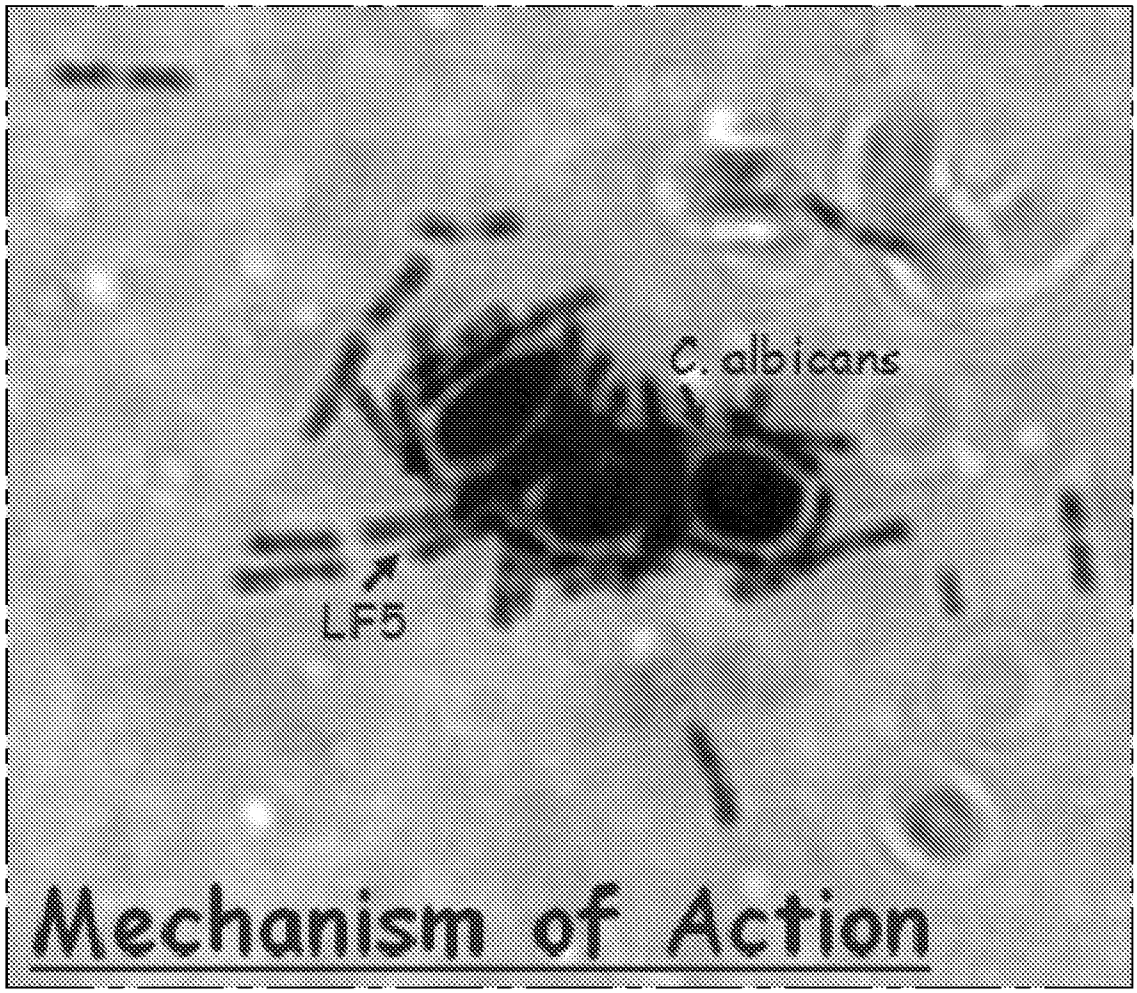


FIG.4

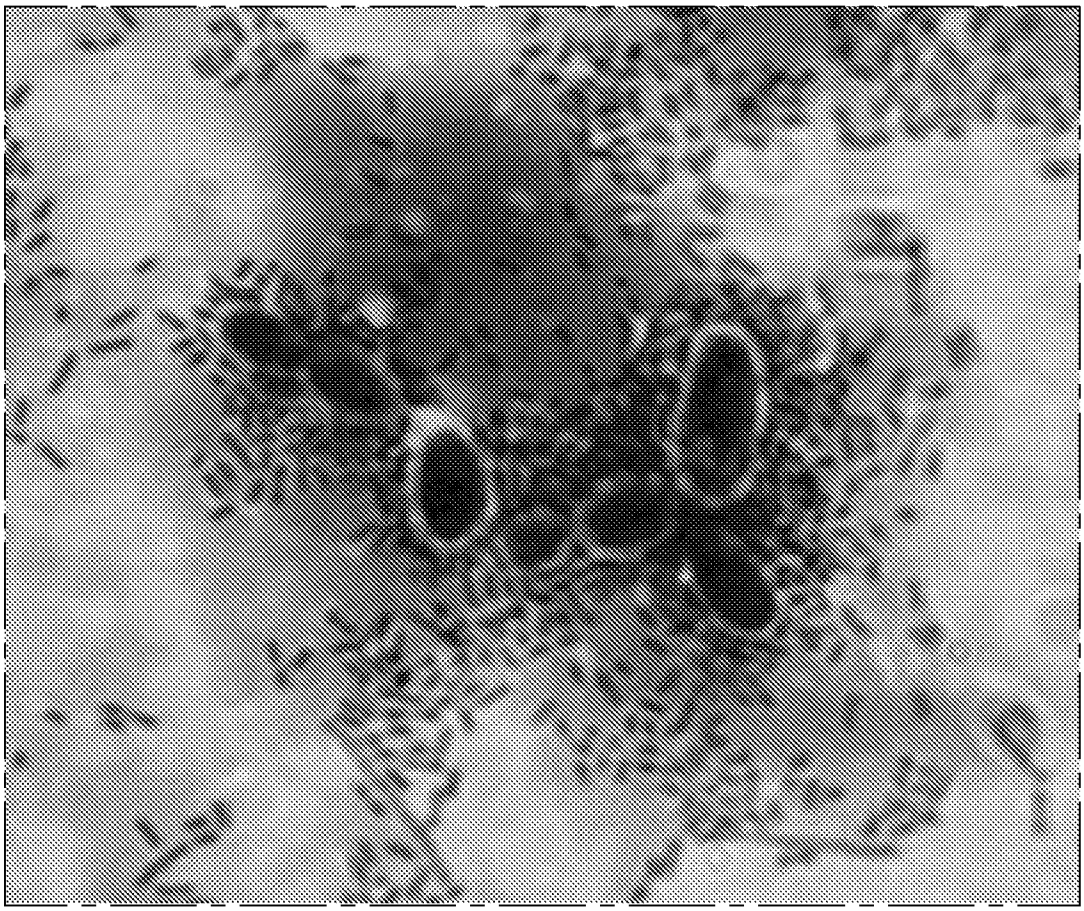


FIG.5

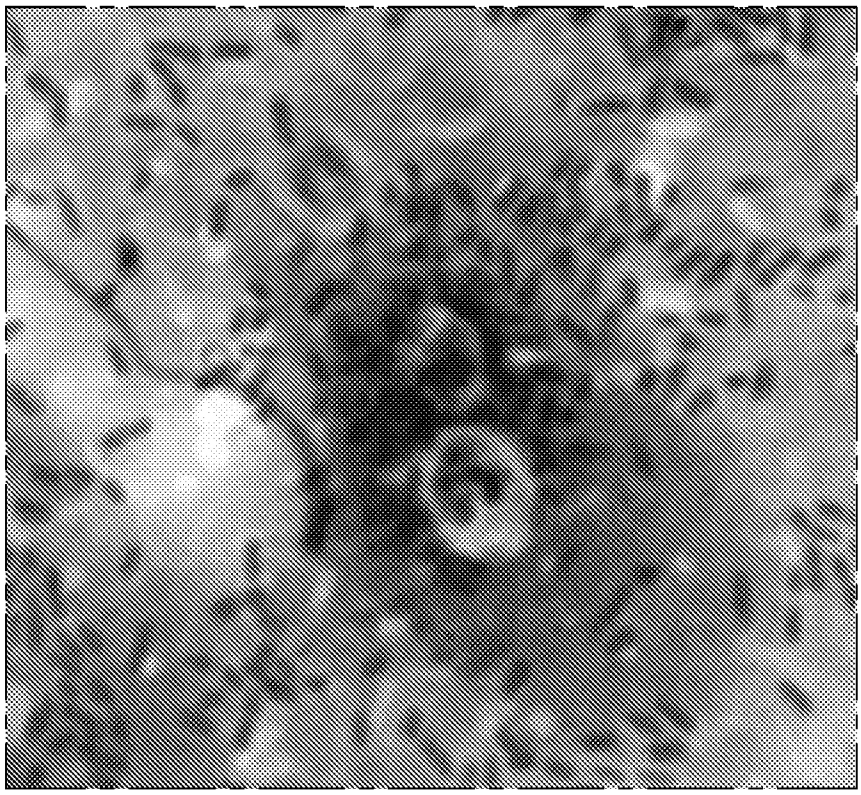
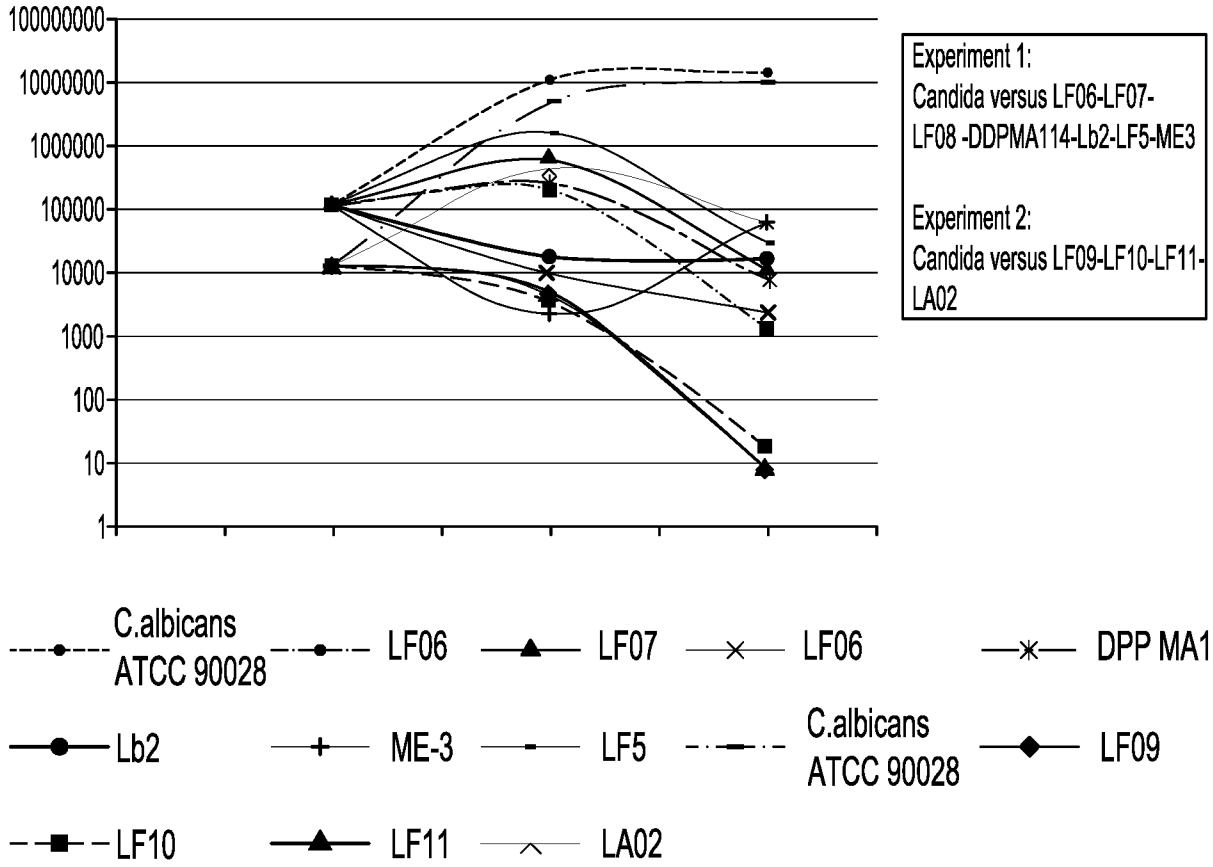


FIG.6



Inhibition of C.albicans ATCC 90028: Graphs of overall data

Graph 5

	cfu/ml	24h	48h	LF6 / T24	LF6/ T48	LF7 / T24	LF7/ T48	LF8 / T24	LF8/ T48	DPPMA114 / T24	DPPMA114 / T48
C. albicans ATCC 90028	$100x10^3$	$10x10^6$	$14x10^6$	$21x10^2$	$14x10^2$	$59x10^4$	$111x10^2$	$1x10^4$	$232x10^3$	$25x10^2$	$83x10^2$
C. albicans ATCC 10231	$55x10^3$	$5x10^6$	$8x10^6$	$78x10^2$	$49x10^2$	$3x10^5$	$78x10^2$	$46x10^2$	$177x10^2$	$357x10^2$	$15x10^2$
C. glabrata ATCC 2001	$450x10^3$	$55x10^6$	$70x10^6$	$14x10^4$	$33x10^2$	$15x10^4$	$47x10^3$	$13x10^2$	$208x10^2$	$13x10^4$	$108x10^2$
C. parapsilosis ATCC 22019	$100x10^3$	$3x10^6$	$5,4x10^6$	$12x10^2$	97	$3x10^4$	$207x10^2$	$74x10^2$	$52x10^2$	$28x10^2$	$99x10^2$
C. krusei ATCC 6258	$160x10^3$	$20x10^6$	$21x10^6$	$9x10^2$	$52x10^4$	$2x10^3$	$29x10^4$	$79x10^3$	$81x10^3$	$8x10^4$	$31x10^4$
C. tropicalis ATCC 750	$110x10^3$	$15x10^6$	$18x10^6$	$19x10^4$	$68x10^4$	$1,4x10^4$	$10x10^4$	$32x10^2$	$222x10^2$	$67x10^2$	$370x10^2$

Experiment 1 continued

Graph 5

	cfu/ml	24h	48h	Lb2 / T24	Lb2/ T48	ME-3/T24	ME-3/T48	LF5/T24	LF5/T48
C. albicans ATCC 90028	$100x10^3$	$10x10^6$	$14x10^8$	$167x10^2$	$156x10^2$	$21x10^2$	$600x10^2$	$157x10^4$	$250x10^2$
C. albicans ATCC 10231	$55x10^3$	$5x10^6$	$8x10^8$	$42x10^4$	$217x10^2$	$77x10^2$	$13x10^2$	$440x10^2$	$72x10^2$
C. glabrata ATCC 2001	$450x10^3$	$55x10^6$	$70x10^8$	$157x10^2$	$3x10^2$	$213x10^2$	180	$100x10^2$	$99x10^2$
C. parapsilosis ATCC 22019	$100x10^3$	$3x10^6$	$5,4x10^8$	$347x10^2$	$60x10^2$	$200x10^2$	1	$21x10^2$	$270x10^2$
C. krusei ATCC 6258	$160x10^3$	$20x10^6$	$21x10^8$	$28x10^4$	$28x10^4$	$11x10^4$	$42x10^4$	$61x10^4$	$99x10^4$
C. tropicalis ATCC 750	$110x10^3$	$15x10^6$	$18x10^8$	$5x10^4$	$59x10^4$	$270x10^2$	$10x10^4$	$15x10^2$	$30x10^4$

Experiment 1
Graph 5

				LF 09		LF 10		LF 11		LA 02	
	cfu/ml	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
C. albicans ATCC 90028	$13x10^3$	$48x10^5$	$68x10^5$	4660	8	4000	20	4480	9	470000	59000
C. albicans ATCC 10231	$25x10^3$	$386x10^4$	$45x10^5$	440	2	250	7	520	0	260000	61000
C. glabrata ATCC 2001	$10x10^3$	$12x10^6$	$34x10^6$	6500	320	12600	137	61000	12	2280000	1170000
C. parapsilosis ATCC 22019	$30x10^3$	$60x10^4$	$35x10^5$	8900	8500	2700	1100	12000	1100	260000	120000
C. krusei ATCC 6258	$19x10^3$	$66x10^4$	$27x10^5$	7200	3400	2400	900	600	3	280000	1500

Experiment 2

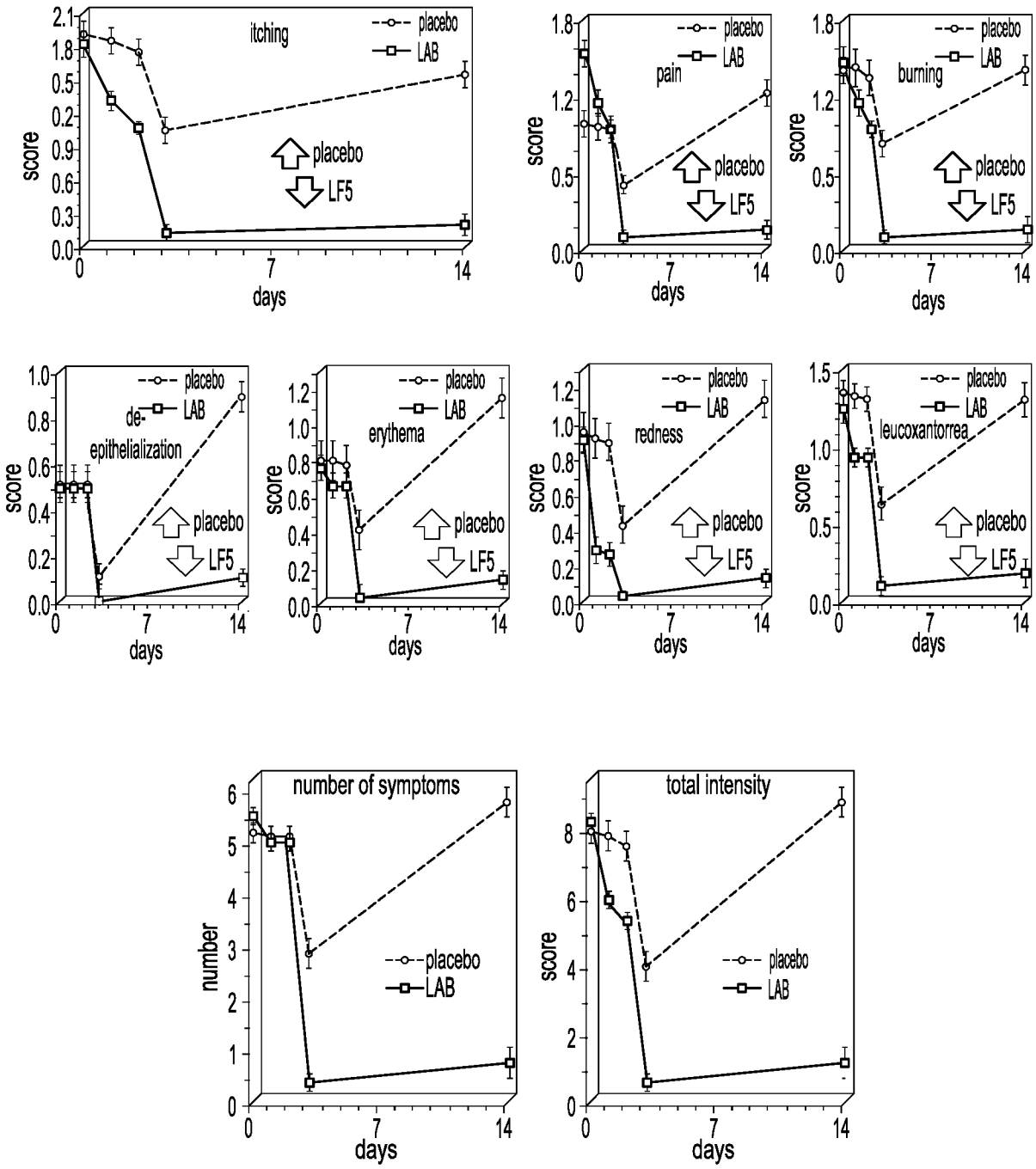
Graph 5

24H	C. albicans ATCC 90028	C. albicans ATCC 10231	C. glabrata ATCC 2001	C. parapsilosis ATCC 22019	C. tropicalis ATCC 750	C. kruzei ATCC 6258
LF5	**99.9843000**	**99.9877778**	**99.9761905**	**99.9790000**	**99.9833333**	**99.9618750**
LF6	**99.7900000**	**99.9980500**	**99.9953333**	**99.9997000**	**99.9881250**	**99.9952632**
LF7	**99.4100000**	**99.9925000**	**99.9950000**	**99.9925000**	**99.9991250**	**99.9989474**
LF8	**99.9900000**	**99.9988500**	**99.9956667**	**99.9981500**	**99.9998000**	**99.9584211**
ME-3	**99.9997900**	**99.9978611**	**99.9994929**	**99.8000000**	**99.9970000**	**99.9931250**
DPPMA114	**99.9791667**	**99.9958000**	**99.9983544**	**99.9972000**	**99.9995533**	**99.9963636**
Lb2	**99.9986083**	**99.9505882**	**99.9998013**	**99.9653000**	**99.9966667**	**99.9872727**
LF09	**99.9990292**	**99.9998860**	**99.9994583**	**99.9851667**	**99.9238095**	**99.9890909**
LF10	**99.9991667**	**99.9999352**	**99.9989500**	**99.9955000**	**99.9823810**	**99.9963636**
LF11	**99.9990667**	**99.9998653**	**99.9949167**	**99.9800000**	**99.9925714**	**99.9990909**
LA02	**99.5833333**	**98.4455959**	**99.9708333**	**99.3333333**	**99.9923810**	**99.8181818**

48H	C. albicans ATCC 90028	C. albicans ATCC 10231	C. glabrata ATCC 2001	C. parapsilosis ATCC 22019	C. tropicalis ATCC 750	C. kruzei ATCC 6258
LF5	**99.9997500**	**99.9989714**	**99.9997848**	**99.9754545**	**99.9700000**	**99.9238462**
LF6	**99.9998923**	**99.9990200**	**99.9999484**	**99.9999820**	**99.9622222**	**99.9752381**
LF7	**99.9991462**	**99.9984400**	**99.9999266**	**99.9961667**	**99.9944444**	**99.9861905**
LF8	**99.9982154**	**99.9964600**	**99.9996750**	**99.9990370**	**99.9987667**	**99.9614286**
ME-3	**99.9940000**	**99.9998143**	**99.9999961**	**99.9999991**	**99.9900000**	**99.9676923**
DPPMA114	**99.9994813**	**99.9998500**	**99.9998615**	**99.9945000**	**99.9969167**	**99.9845000**
Lb2	**99.9990250**	**99.9978300**	**99.9999962**	**99.9966667**	**99.9508333**	**99.9860000**
LF09	**99.9999991**	**99.9999996**	**99.9999906**	**99.9975714**	**99.9980000**	**99.9987407**
LF10	**99.9999977**	**99.9999984**	**99.9999960**	**99.9996857**	**99.9999991**	**99.9996667**
LF11	**99.9999990**	**100.0000000**	**99.9999996**	**99.9996857**	**99.9986957**	**99.9999989**
LA02	**99.9932955**	**99.9864444**	**99.9655882**	**99.9657143**	**99.9913043**	**99.9994444**

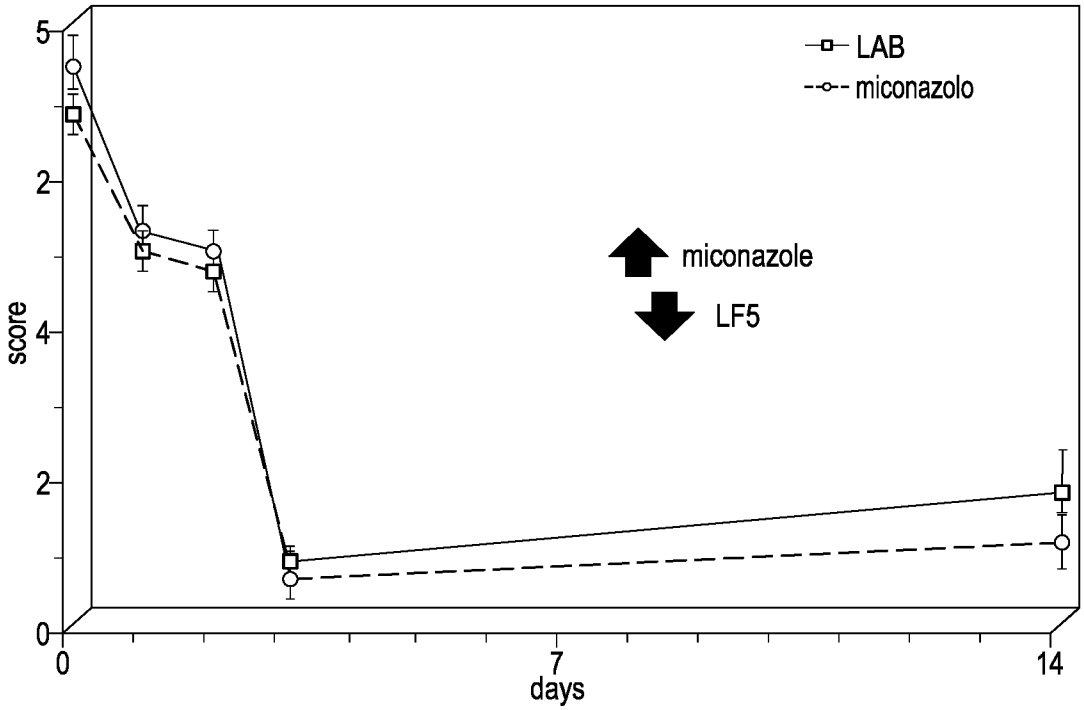
Experiments on the strains of bacteria *L.Fermentum*
with the MICs at 24 and 48 hours

Table 11



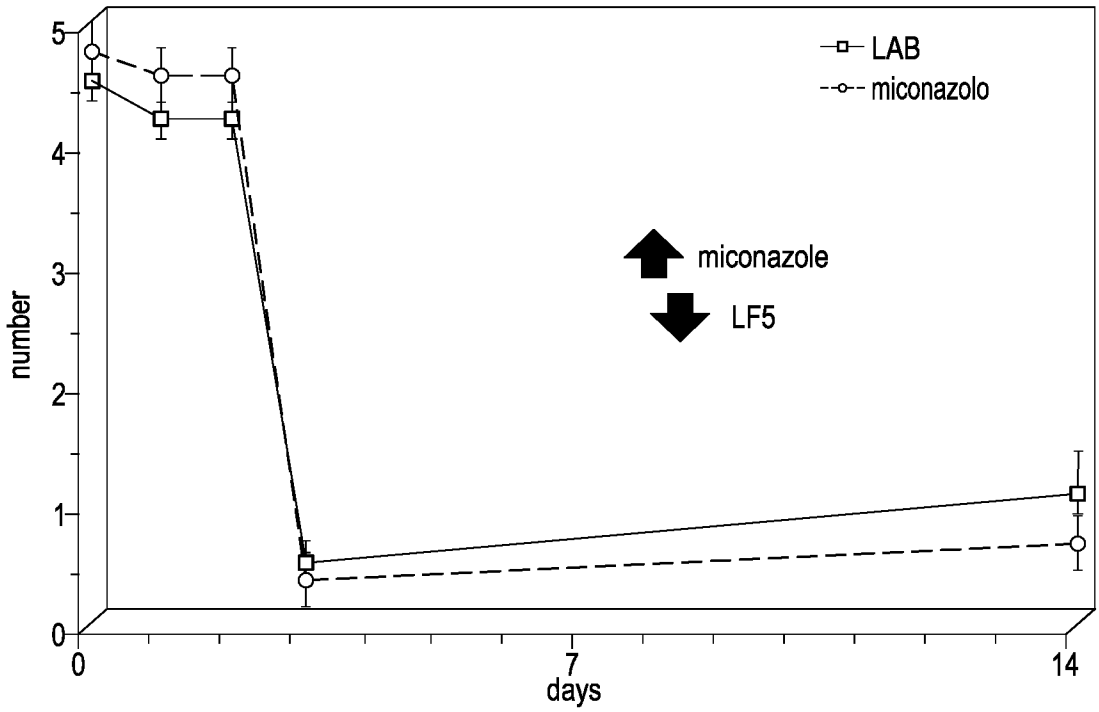
Clinical study for the efficacy and tolerability of LF5 compared with placebo in patients with *Candida albicans*.

FIG.7



Trend of the total average number of symptoms and signs (+ESM) related to the fungal vaginal colonization, during and after treatment with LF5 or miconazole

FIG.8a



Trend of the total average intensity of symptoms and sign (+ESM) related to the fungal vaginal colonization, during and after treatment with LF5 or miconazole

FIG.8b

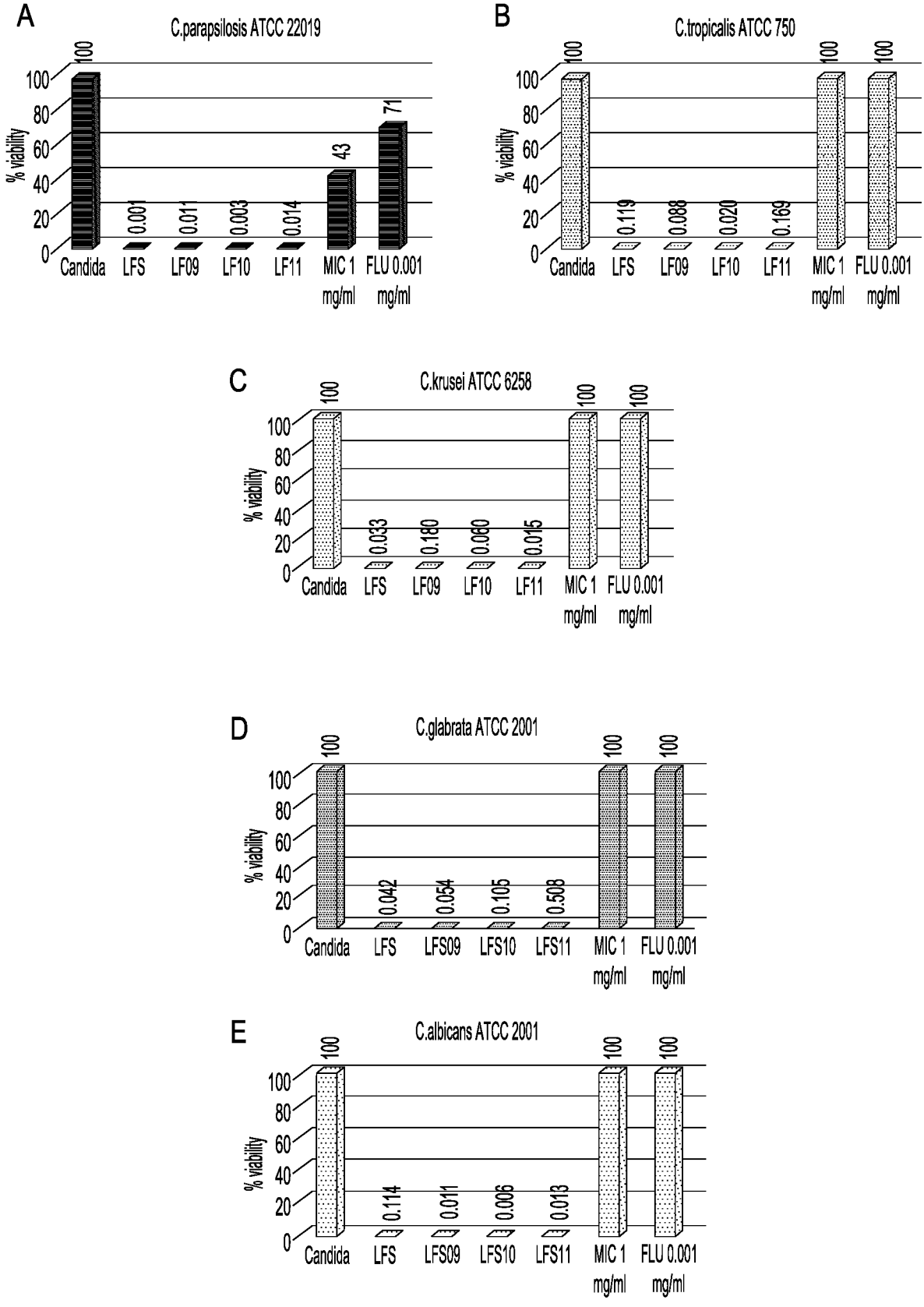
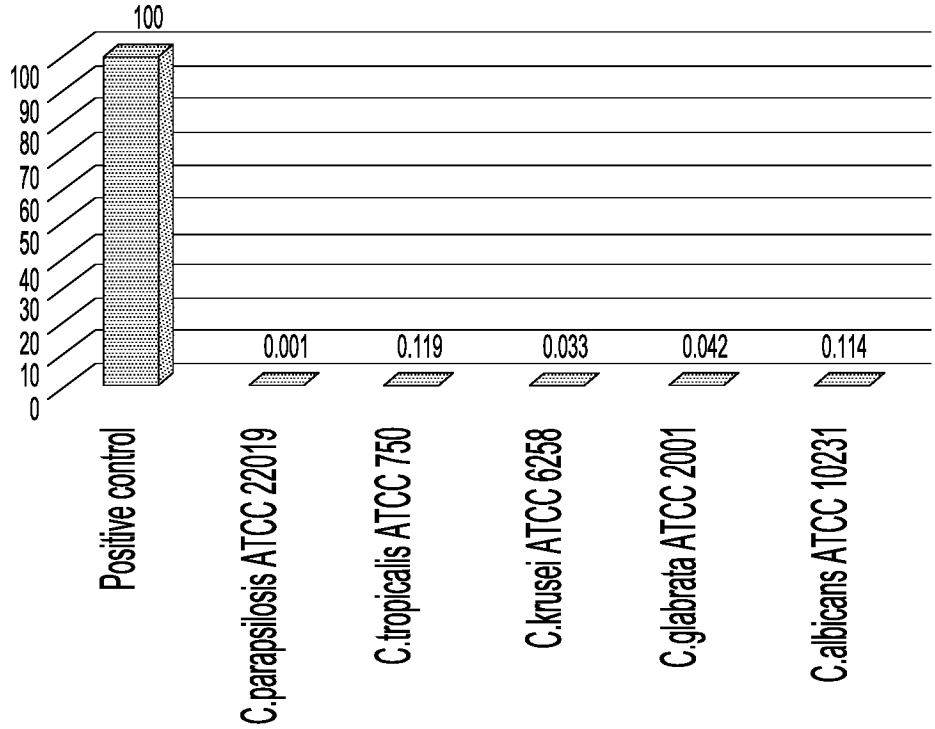


FIG.9

AMENDED SHEET (ARTICLE 19)



Antimicrobial activity of *Lactobacillus fermentum* LFS against 5 species of *Candida:*
(A) *Candida parapsilosis* (ATCC 22019);
(B) *Candida tropicalis* (ATCC 750);
(C) *Candida krusei* (ATCC 6258);
(D) *Candida glabrata* (ATCC 2001);
and (E) *Candida albicans* (ATCC 10231).
Positive control is represented by one of the *Candida* strain grown alone.

FIG.10

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/051711

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/747 A61P15/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	FRANCESCA DEIDDA ET AL: "In Vitro Activity of Lactobacillus fermentum LF5 Against Different Candida Species and Gardnerella vaginalis", JOURNAL OF CLINICAL GASTROENTEROLOGY., vol. 50, 1 November 2016 (2016-11-01), pages S168-S170, XP055324073, US ISSN: 0192-0790, DOI: 10.1097/MCG.0000000000000692 the whole document ----- -/--	1-10

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2017	10/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schnack, Anne

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 2012/101500 A1 (PROBIOTICAL SPA [IT]; MOGNA GIOVANNI [IT]; STROZZI GIAN PAOLO [IT]; MO) 2 August 2012 (2012-08-02) claims 8-10	1-10
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Y	page 17 - page 19	1-10

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Information on patent family members

International application No
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