

DESCRIPTION of the invention entitled:

"*Lactcaseibacillus rhamnosus* DSM 33960 and its use in a method for the prevention and treatment of urogenital tract dysbiosis"

5 FIELD OF INVENTION

It is an object of the present invention the *Lactcaseibacillus rhamnosus* DSM 33960 bacterial strain, and its use in a method for the prevention and treatment of conditions of dysbiosis of the urogenital tract, particularly the female urogenital tract, preferably vaginal dysbiosis.

TECHNICAL FIELD

10 The vaginal microbiota, although characterized by a few microbial species, is a dynamic ecosystem subject to change throughout a woman's life, from birth to pre- and post-menopausal phases. In addition, intrinsic factors (such as race, immune system imbalance, genetic susceptibility, etc.) and extrinsic factors (such as diet rich in vitamins and folic acid, use of oral contraceptives, sexual behaviors, hygiene habits, use of antibiotics or immunosuppressive therapies) can affect the balance of the vaginal microbiota, altering it.

15 Alterations in the vaginal microbiota (vaginal dysbiosis) are associated with many diseases such as, for example, urogenital tract infections (e.g., by *Candida*) reproductive dysfunction, obstetric complications, and cancer. Traditionally, vaginal dysbiosis is treated with antimicrobial drugs (such as metronidazole and clindamycin). However, antimicrobial drugs have several undesirable effects, such as short-term recurrence and promotion of antibiotic resistance. For these reasons, over time, alternative therapeutic strategies have been studied that can
20 modulate the vaginal microbiota and correct its imbalances. In this context, the administration of probiotics, either alone or to complement antibiotic therapy, has gained increasing attention. However, probiotics currently known in the art frequently present host specificity problems and, consequently, poor efficacy in counteracting vaginal dysbiosis and related diseases. In addition, the probiotics currently known in the art are often found to be insufficiently safe and, therefore, unsuitable for use in medical field, for example, in the prevention or treatment of
25 vaginal dysbiosis. In fact, although most probiotics currently known in the art are recognized as generally safe, both safety and probiotic characteristics are specific to each strain and cannot be considered as representative characteristics of the entire species to which the strain belongs.

In addition, known probiotic strains frequently turn out not to be usable in combination with antibiotic therapies.

International patent application WO 2022/009170 A1, as well as the scientific publication Pino, A., Rapisarda, A.M.C., Vitale, S.G. et al. "A clinical pilot study on the effect of the probiotic *Lactcaseibacillus rhamnosus* TOM 22.8 strain in women with vaginal dysbiosis," and related "Supplementary information," Sci Rep 11, 2592 (2021),
30 <https://doi.org/10.1038/s41598-021-81931-z>, disclose a strain of *Lactcaseibacillus rhamnosus* (strain TOM 22.8 - DSM 33500) for use in women with vaginal dysbiosis.

Therefore, there is still a need for bacterial strains, particularly probiotic strains, that can prevent and/or
35 counteract vaginal dysbiosis.

In particular, it is now necessary to have available bacterial strains (probiotics) capable of acting at the level of the vaginal microbiota in order to prevent and/or counteract imbalances in this microbiota, and related pathologies such as, for example, infections of the urogenital tract.

5 Purposes of the invention

It is a purpose of the present invention to make available an isolated bacterial strain of human origin.

Another purpose of the present invention is to make available an isolated bacterial strain for use in a method for the prevention and treatment of urogenital tract dysbiosis, preferably vaginal dysbiosis.

Still purpose of the present invention is to make available an isolated bacterial strain that is effective in preventing
10 and/or treating diseases or conditions related to dysbiosis of the urogenital tract, for example, infections of the urogenital tract, particularly vaginal infections.

Further purpose of the present invention is to make available a probiotic bacterial strain that can be used, for example, in rebalancing the vaginal microbiota, effectively and safely, and that is host-specific.

These and other purposes are achieved by the object of the present invention, which provides an isolated
15 bacterial strain suitable for use in the prevention and/or treatment of dysbiosis of the urogenital tract, particularly vaginal dysbiosis.

Description of the invention

It is an object of the present invention a new isolated microorganism, specifically a new bacterial strain belonging
20 to the genus *Lacticaseibacillus*, not genetically modified, named *Lacticaseibacillus rhamnosus* CA15, deposited by ProBioEtna Srl on July 28, 2021, at the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ), under deposit number DSM 33960.

The microorganism (strain) according to the present invention, i.e., *Lacticaseibacillus rhamnosus* CA15 DSM 33960 (also referred to in this description as "*Lacticaseibacillus rhamnosus* DSM 33960" or, more simply, "DSM
25 33960") was isolated from the vaginal ecosystem of a healthy and asymptomatic Italian woman afferent to the Department of Obstetrics and Gynecology of the A.O.U. of the Policlinico - Vittorio Emanuele P.O. G. Rodolico of the University of Catania. To isolate the strain of the invention, vaginal exudate samples were processed as previously reported (Pino et al., 2021; 2019). Vaginal exudate, taken using appropriate sterile pads provided with transport medium, was analyzed as follows. After removing the cells, employing sterile phosphate-buffered saline
30 (PBS), serial dilutions were made and plated in de Man Rogosa and Sharp growth medium (MRS, Oxoid, Milan, Italy). The plates were incubated at 35-37°C for 40-48 h. In the context of the present invention "h" may mean hour or hours, intended as time, depending on the context.

Lacticaseibacillus rhamnosus CA15 DSM 33960 strain has a bacillary morphology and is Gram positive. The microorganism of the invention is catalase negative, that is, it does not have the enzyme catalase, which can
35 hydrolyze hydrogen peroxide.

DSM 33960 strain is, in addition, unable to produce spores and does not exhibit motility.

Advantageously, the strain of the invention can be used as a medicament. Therefore, an object of the present invention is *Lacticaseibacillus rhamnosus* DSM 33960 strain for use as a medicament.

Surprisingly, high antagonistic activity of *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain, according to the invention, could be observed toward a broad spectrum of potentially pathogenic bacteria and yeasts such as, for example, *Gardnerella vaginalis*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, and *Streptococcus agalactiae*.

In addition to the above-mentioned antagonistic activity, the Applicant was able to observe additional advantageous features of the strain of the invention such as, for example, high anti-inflammatory and antioxidant activity, the ability to adhere to the intestinal mucosa, the ability to produce exopolysaccharides (EPS), the ability to produce biofilms, and the ability to antagonize biofilm formation by different *Candida* species as well as to inhibit the adhesion of a variety of pathogens and counteract the development of a variety of adherent pathogens. These characteristics make the strain of the invention particularly suitable for use in the prevention and/or treatment of dysbiosis of the microbiota of the urogenital tract, particularly of the female urogenital tract, preferably of the dysbiosis of the vaginal microbiota. In other words, the strain of the invention can be, advantageously, used in the prevention and/or treatment of alterations in the microbiota of the urogenital tract, preferably the vaginal microbiota.

Therefore, according to one of its aspects, it is an object of the invention the *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain for use in the prevention and/or treatment of dysbiosis of the microbiota of the urogenital tract, preferably vaginal dysbiosis.

In this description, the term "dysbiosis" refers to a condition of altered homeostasis of the microbiota associated with a specific microbial ecosystem of the human body. The terms "urogenital tract dysbiosis" and "vaginal dysbiosis" refer to an altered condition of the microbiota of the urogenital tract and vaginal microbiota, respectively, compared with their normal physiological conditions.

According to a further aspect, it is an object of the invention the *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain for use in a method for the prevention and/or treatment of diseases related to dysbiosis of the urogenital tract microbiota, particularly the female urogenital tract microbiota, preferably the vaginal microbiota.

Examples of pathologies related to dysbiosis of the vaginal microbiota are vaginal infections, vaginal inflammation, bacterial vaginosis (BV) and recurrences thereof, vulvovaginal candidiasis (VVC), and sexually transmitted bacterial infections carried, for example, by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*.

In particular, the *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain can be used for the prevention and/or treatment of urogenital tract infections, particularly female urogenital tract infections, preferably vaginal infections. In embodiments, the urogenital tract infections, particularly vaginal infections, can be selected from infections due to one or more microorganisms such as *Gardnerella vaginalis*, *Escherichia coli*, *Staphylococcus aureus*, *Candida* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, and *Streptococcus agalactiae* and combinations thereof. Preferably, *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain can be used for the

- prevention and/or treatment of urogenital tract infections, urogenital tract inflammation, bacterial vaginosis (BV) and recurrences thereof, vulvovaginal candidiasis (VVC), mixed dysbiosis, and group B streptococcal infections. In embodiments, the strain of the invention can be used to prevent and/or treat bacterial vaginosis (BV) and recurrences thereof, and/or vulvovaginal candidiasis (VVC), group B streptococci (GBS) infections, urinary tract
- 5 infections caused by *Pseudomonas aeruginosa* and *Escherichia coli*.
Lactiseibacillus rhamnosus CA15 DSM 33960 strain can be, advantageously, used in the prevention and/or treatment of inflammation of the urogenital tract, particularly inflammation of the female urogenital tract, preferably vaginal inflammation, e.g., inflammation due to *Gardnerella vaginalis*, *Escherichia coli*, *Candida* spp., *Pseudomonas aeruginosa*, and *Streptococcus agalactiae*.
- 10 In embodiments, the strain of the invention can be administered orally (i.e., gastroenterally) and topically. Preferably, the DSM 33960 strain of the invention is administered orally.
In fact, it was surprisingly observed that the strain of the invention exhibits a high ability to survive stressful conditions in the gastrointestinal (GI) tract. In particular, it was observed that the strain of the invention exhibits high resistance to lysozyme (present, for example, in saliva), bile salts, gastric juice and acidic pH.
- 15 When administered orally, *Lactiseibacillus rhamnosus* CA15 DSM 33960 strain can be administered as powders or granules, such as contained in a tablet, or as a swallowable capsule.
In embodiments, the strain of the invention can be administered in dried or freeze-dried form.
Advantageously, *L. rhamnosus* DSM 33960 strain does not show resistance to the antibiotics suggested by EFSA (European Food Safety Authority), while resistance has been detected to metronidazole, clotrimazole, and boric
- 20 acid, antimicrobials routinely used for the treatment of urogenital tract infections, particularly vaginal infections such as, for example, bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC).
In addition, the *L. rhamnosus* DSM 33960 strain also showed resistance to fluconazole (an antifungal used to treat fungal infections that can also proliferate vaginally).
Therefore, DSM 33960 strain can be advantageously used as an adjuvant to antimicrobial treatment, particularly
- 25 antibiotic/antimycotic treatment, of urogenital tract infections, particularly female urogenital tract infections, preferably vaginal infections.
In this description, the term "adjuvant" refers to the ability to enhance or complement the action of treatments that are the therapy of choice.
In embodiments, the strain of the invention can be administered in combination with one or more antimicrobial
- 30 agents. Preferably, the strain of the invention can be administered in combination with one or more antimicrobial agents selected from metronidazole, clotrimazole, boric acid, fluconazole and mixtures thereof.
According to this description, the phrase "administered in combination with one or more antimicrobial agents" refers to the combined use of the strain of the invention with one or more antimicrobial agents, particularly for preventive and/or therapeutic purposes. The strain of the invention can be administered together with the
- 35 antimicrobial agents or separately to the antimicrobial agents. For example, the strain of the invention can be administered before or after or concurrently with the administration of antimicrobial agents.
The strain of the invention has several advantages over probiotic microorganisms known in the art.

In fact, the strain of the present invention can be used for the prevention and/or treatment of dysbiosis of the urogenital tract, particularly the female urogenital tract, preferably vaginal dysbiosis, effectively and safely for the host.

The strain of the invention produces both H₂O₂ and lactic acid. This characteristic makes it particularly effective as an antagonist against many pathogenic microorganisms of the urogenital tract, particularly vaginal pathogens.

In addition, DSM 33960 strain showed the ability to adhere to Caco-2 (intestinal epithelium model cells) HT29 (grade II colon adenocarcinoma cells) and VK2/E6E7 (vaginal epithelial cell lines) cell lines. In addition, the strain of the invention showed hydrophobicity, auto-aggregation ability and ability to co-aggregate with pathogens. In order to be able to exert beneficial health effect to the host, microbial strains with probiotic potential should be able to adhere to and colonize the gastro-intestinal tract, interacting with resident bacteria. Several mechanisms are involved in the process of bacterial adhesion, which depends on intra- and extra-cellular properties. Auto-aggregation, intrinsic to specific microbial strains, is known to increase the potential for colonization while hydrophobicity, related to the presence of hydrophobic molecules on the cell surface of the strain, amplifies its ability to adhere to the host intestinal mucosa. In addition, the ability to co-aggregate with pathogens is considered a hallmark of specific probiotic strains, as it promotes the formation of a physical-chemical barrier that prevents colonization by pathogenic microorganisms. Without wishing to bind to a specific explanation, it has been hypothesized that these capabilities enable the strain of the invention to inhibit the growth and adhesion to the vaginal epithelium of pathogens, making it particularly effective in exerting a protective role for the urogenital tract, particularly the vagina.

It has also been observed that DSM 33960 strain produces exopolysaccharides (EPS) associated with a variety of health-promoting functions.

In addition, *L. rhamnosus* DSM 33960 strain exhibits important antioxidant and anti-inflammatory capacities to prevent tissue damage due to oxidative stress, and the development of related diseases, and to stimulate the immune system response in an anti-inflammatory manner as a response to pro-inflammatory stimuli.

DSM 33960 strain also showed a high survival rate at low pH, in the presence of lysozyme, bile salts and during *in vitro* simulated gastrointestinal digestion. These characteristics make the strain of the invention particularly suitable for oral administration.

The present invention will now be illustrated through the following experimental section, which is to be considered purely illustrative and not limiting.

Brief description of the drawings

Figure 1: Genetic fingerprint via PFGE ("Pulsed-field gel electrophoresis"). PFGE profiles of genomic DNA digested with *Ascl* restriction enzyme of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain. M: Lambda PFG marker (NEB, US).

Figure 2: Results of an ABTS assay, designed to measure the antioxidant activity of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, using 2,2-azino di-(3-ethylbenzthiazoline sulfonate) (ABTS) substrate. Results are expressed as absorbance values at 500nm.

Figure 3: Adhesion of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 and *Lactocaseibacillus rhamnosus* GG strains to human intestinal mucus glycoproteins. The results are expressed as mean percent adhesion and standard deviation of three replicates each consisting of six technical replicates.

Experimental section

Example 1 - Isolation of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, phenotypic and genotypic characterization

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain was isolated from the vaginal ecosystem of a healthy patient, afferent at the Gynecologic Obstetric Clinic of the A.O.U. of Policlinico - Vittorio Emanuele P.O. G. Rodolico of the University of Catania, employing De Man, Rogosa and Sharpe (MRS) growth medium. A pure culture of the strain, obtained by purification smears, was subjected to phenotypic characterization by microscopic observation, catalase test, Gram staining, evaluation of spore-producing ability and motility. The strain was stored, in pure culture, at -80 °C using a 20% glycerol (v/v) solution.

The phenotypic characteristics of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain are shown in Table 1.

Table 1					
Phenotypic characterization					
	<u>Morphology</u> <u>under the</u> <u>microscope</u>	<u>Gram staining</u>	<u>Catalase</u>	<u>Ability to produce spores</u>	<u>Motility</u>
DSM 33960	Bacillary	Gram positive	Negative	Absent	Absent

The strain was subjected to genotypic characterization by: PCR species-specific *tuf*-gene , species-level characterization by 16S rDNA gene sequencing, and strain-level typing by Pulsed Field Gel Electrophoresis (PFGE) analysis, according to the requirements of the FAO/WHO 2002 guidelines.

A culture of *L. rhamnosus* DSM 33960 strain, in stationary growth phase, was subjected to genomic DNA extraction using a commercial kit (Qiagen GmbH, Hilden, Germany). The genomic DNA was subsequently amplified by conventional PCR using P1-P4 primers targeting the V1-V3 regions of the 16S rDNA gene. The resulting amplicon was sequenced and the species was identified through the BLAST database "Nucleotide collection (nt/nt)" (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identification was considered unambiguous at >99% sequence homology. Strain-level typing was performed using pulsed field electrophoresis (PFGE). Plugs preparation, DNA extraction, and enzymatic restriction were performed using the CHEF Bacterial Genomic DNA Plug Kits (Bio-Rad, UK) according to the manufacturer's protocol indicated for Gram-positive bacteria. After digestion with *AscI* restriction enzyme at 37°C for 18 h, electrophoresis was performed using CHEF DR II (Bio-

Rad, Milan, Italy) in 1 % Megabase agarose (Bio-Rad, Milan, Italy) in 0.5X TBE buffer. The gels were then stained with 1X Atlas ClearSight DNA Stain (BIOATLAS OÜ, Estonia) for one hour and visualized under UV light. The results of the PFGE analysis are shown in Figure 1.

5 Example 2 - Safety Performance

According to the guidelines for probiotics and prebiotics, prepared by the Ministry of Health, *Lactcaseibacillus rhamnsous* CA15 DSM 33960 strain was evaluated for safety requirements. In detail, the following were studied: hemolytic activity, ability to produce DNase and gelatinase, ability to degrade mucin, ability to produce biogenic amines as well as antibiotic resistance against a variety of antibiotics whose break-point values have been established by the European Food Safety Authority (EFSA). In addition, resistance/susceptibility to antimicrobial agents used in common clinical practice (metronidazole, clotrimazole and boric acid) was tested. Below in detail are the characterizations carried out on the strain under study.

2.1. Hemolytic activity.

15 *Lactcaseibacillus rhamnsous* CA15 DSM 33960 strain was revitalized in MRS broth medium and incubated at 37°C for 16-18 hours. The cell culture in exponential growth phase was transferred by smear to Blood Agar plates containing 5% defibrinated mutton blood (Biolife, Milan, Italy) and incubated at 37°C for 24-48 hours. Hemolytic activity was determined visually and distinguished as β -hemolysis, α -hemolysis, or γ -hemolysis based on the presence of clear zones, green halos, or no zones around the colonies, respectively. The β -hemolytic strain *Streptococcus pyogenes* ATCC 19615 and the α -hemolytic strain *Streptococcus pneumoniae* ATCC 6303 were used as positive controls. The results, shown in Table 2, were expressed as "+" (presence of hemolytic activity) and "-" (absence of hemolytic activity). The tested strain showed no hemolytic activity.

Table 2	
Activity tested	<i>L. rhamnsous</i> CA15 DSM 33960
Hemolytic activity	-

25 2.2. Ability to produce DNase and gelatinase

DNase production was tested by transferring 5 μ l of cell culture in exponential growth phase onto DNase agar plates (Oxoid). After incubation at 37 °C for 48 h, the plates were treated with HCl 1 N for 5 min. The presence of clear areas around the colonies is considered as an indicator of DNase production positivity.

Gelatinase production was evaluated using gelatin agar plates (30 g/L gelatin, 5 g/L peptone, 3 g/L yeast extract, and 17 g/L agar). After incubation at 37 °C for 48 h, the surface of the plates was treated with saturated ammonium sulfate (Merck, Milan). The presence of clear areas around the colonies is considered as an indicator of positive gelatinase activity.

For both tests, the results, shown in Table 3, were expressed as: "-" (absence of activity), "+" (presence of activity). The tested strain showed no ability to produce DNase and gelatinase.

Table 3	
Tested enzyme activity	<i>L. rhamnosus</i> CA15 DSM 33960
DNAse	-
Gelatinase	-

2.3. Ability to degrade mucin

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to degrade mucin was evaluated using a suitable growth medium modified by the addition of 0.5% (w/v) of porcine stomach type III (Sigma-Aldrich, St. Louis, MO, USA). The cell culture in exponential growth phase was transferred into the aforementioned growth medium, and the plates were incubated at 37 °C for 72 h. Next, the plates were stained with 0.1% (w/v) starch black (Merck, Milan) in acetic acid 3.5 M for 30 min, then washed with acetic acid 1.2 M (Merck, Milan). The presence of halos around the colonies was considered as a positive result.

The results, shown in Table 4, were expressed as: "-" (absence of activity), "+" (presence of activity). The tested strain showed no ability to degrade mucin.

Table 4	
Activity tested	DSM 33960
Degradation of mucin	-

2.4. Production of biogenic amines

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain was reactivated by 2% inoculation in de Man Rogosa and Sharp broth medium (MRS, Oxoid, Milan, Italy) and incubated at 37°C overnight. The resulting biomass was subcultured five times in liquid medium containing 0.005% pyridoxal-5'-phosphate (Sigma Aldrich St. Louis, MO, USA) and biogenic amine production was stimulated by adding 0.1% histidine, 0.1% lysine, 0.1% tyrosine or 0.1% ornithine (Sigma Aldrich, St. Louis, MO, USA). Biogenic amine extraction and estimation protocols were performed as follows. Specifically, the extracted and derivatized samples were separated by HPLC chromatography (Shimadzu Nexera XL UHPLC system equipped with an SPD M30A diode array detector) using a Discovery HS C18 250 mm x 46 mm x 5 µm column (Sigma Aldrich, St. Louis, MO, USA) applying a low pressure gradient of acetonitrile and water. Separation temperature was kept constant at 26 °C to allow better reproducibility of results. Six-point standard curves were generated and considered suitable if they had a correlation value $R^2 > 0.99$. The samples were analyzed in duplicate, and the results are shown in Table 5 and expressed as mean and standard deviation.

Table 5	
Biogenic amines (g/l)	
Histamine	nd
Spermine	nd
Tyramine	0.003
Putrescine	nd
Cadaverine	nd

Quantification, performed according to the procedure described in ISO19343:2017, found that the tested strain does not produce relevant amounts of the assayed biogenic amines.

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2.5. Antibiotic susceptibility and MIC determination.

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain was tested in order to determine its resistance or susceptibility to certain antibiotics, in accordance with the European Food Safety Authority (EFSA) report, by minimum inhibitory concentration (MIC) assessment using EFSA's (2018) suggested cut-offs as a reference. For each antibiotic tested, the strain was classified as resistant (R) or susceptible (S). In addition to the antibiotics suggested by EFSA, the strain was tested for resistance/susceptibility to some antimicrobial agents used in common clinical practice (metronidazole, clotrimazole, and boric acid).

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As can be seen from the results, shown in Table 6, *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain is susceptible to all antibiotics suggested by EFSA.

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As can be seen from the results, shown in Table 7, *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain showed resistance against metronidazole, clotrimazole and boric acid. The strain also did not show genome-wide antibiotic resistance genes. In Table 6, the MIC and breakpoint values (threshold values that allow a strain to be classified as susceptible or resistant) for each antibiotic tested, expressed in µg/ml, are shown.

Table 6			
Activity tested		DSM 33960	Breakpoints
Antibiotic resistance	Ampicillin	2	4
	Chloramphenicol	1	4
	Clindamycin	0.25	1
	Erythromycin	0.5	1
	Gentamicin	8	16
	Kanamycin	32	64
	Streptomycin	16	32
	Tetracycline	4	8

Table 7		
Activity tested		DSM 33960
Resistance to antimicrobial agents	Boric acid	>10000
	Clotrimazole	>256
	Metronidazole	>600

In a survival test during gastrointestinal transit using the dynamic SHIME system, *L. rhamnosus* DSM 33960 strain also showed resistance to fluconazole (an antifungal used to treat fungal infections that can also proliferate vaginally). See, for more details, section 3.17-C below.

Example 3 - Functional performance

3.1. Antimicrobial activity

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain was tested for antagonistic activity against the potential pathogens *Enterobacter cloacae* DSM 30054, *Enterococcus faecalis* DSM 2570, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 700414, *Escherichia coli* DSM 105393, *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 14243, *Candida parapsilosis* ATCC 90018, *Candida tropicalis* ATCC 13803, *Gardnerella vaginalis* ATCC 14019, *Gardnerella vaginalis* ATCC 14018, *Listeria monocytogenes* DSM 12464, *Proteus mirabilis* DSM 30116, *Pseudomonas aeruginosa* DSM 1117, *Pseudomonas aeruginosa* DSM 3227, *Pseudomonas monteilii* ATCC 700476, *Staphylococcus aureus* ATCC 6538, and *Streptococcus agalactiae* DSM 2134. Antimicrobial activity was evaluated by employing both cells and metabolites produced during cell growth. The test with cells was performed by agar spot test. After incubation, antimicrobial activity was evaluated by measurement of the halo of inhibition obtained. The results, shown in Table 8, were expressed as "-" (no activity); "+" (halo of inhibition <10 mm in diameter); "++" (halo of inhibition between 11 and 20 mm in diameter); "+++" (halo of inhibition >20 mm in diameter). For evaluation of the antimicrobial activity exerted by metabolites produced during cell growth, the supernatant ("CFS," "Cell-free supernatant") obtained after centrifugation and filtration of a cell culture in broth medium was tested before and after neutralization, inactivation by heat, treatment with catalase and proteolytic enzymes.

All experiments were conducted in triplicate.

The strain under study showed a broad spectrum of antimicrobial activity. Based on the treatments, to which CFS was subjected, the antagonistic activity was, in all cases, attributed to the production of organic acids.

In detail, the highest antimicrobial activity was shown against *Candida albicans* ATCC 10231, *Candida krusei* ATCC 14243, *Gardnerella vaginalis* ATCC 14018, and *Streptococcus agalactiae* DSM 2134 (zone of inhibition larger than 20 mm). Zones of inhibition between 11 and 20 mm in diameter were observed against *Candida glabrata* ATCC 90030, *Candida tropicalis* ATCC 13803, *Gardnerella vaginalis* ATCC 14019, *Listeria monocytogenes* DSM 12464, *Pseudomonas monteilii* ATCC 700476, and *Staphylococcus aureus* ATCC 6538. Zones of inhibition having a diameter of less than 10 mm were found against: *Escherichia coli* ATCC 700414,

Candida parapsilosis ATCC 90018, *Pseudomonas aeruginosa* DSM 1117, and *Pseudomonas aeruginosa* DSM 3227.

Table 8		
Activity tested		DSM 33960
Antimicrobial activity	<i>Enterobacter cloacae</i> DSM 30054	-
	<i>Enterococcus faecalis</i> DSM 2570	-
	<i>Escherichia coli</i> ATCC 25922	-
	<i>Escherichia coli</i> ATCC 700414	+
	<i>Escherichia coli</i> DSM 105393	-
	<i>Candida albicans</i> ATCC 10231	+++
	<i>Candida glabrata</i> ATCC 90030	++
	<i>Candida krusei</i> ATCC 14243	+++
	<i>Candida parapsilosis</i> ATCC 90018	+
	<i>Candida tropicalis</i> ATCC 13803	++
	<i>Gardnerella vaginalis</i> ATCC 14019	++
	<i>Gardnerella vaginalis</i> ATCC 14018	+++
	<i>Listeria monocytogenes</i> DSM 12464	++
	<i>Proteus mirabilis</i> DSM 30116	-
	<i>Pseudomonas aeruginosa</i> DSM 1117	+
	<i>Pseudomonas aeruginosa</i> DSM 3227	+
	<i>Pseudomonas monteilii</i> ATCC 700476	++
	<i>Staphylococcus aureus</i> ATCC 6538	++
	<i>Streptococcus agalactiae</i> DSM 2134	+++

5 3.2. Production of hydrogen peroxide

The ability to produce hydrogen peroxide (H₂O₂) was evaluated by growing the strain, previously revitalized in broth medium, on MRS agar containing 0.25 mg/mL of 3,3',5,5'-tetramethylbenzidine and 0.01 mg/mL of horseradish peroxidase. After incubation for 72 h, the plates were exposed to air, and the production of H₂O₂ was evaluated by the appearance of blue staining. The ability to produce hydrogen peroxide (H₂O₂) was evaluated on the basis of the time required for the appearance of blue staining as follows: absent (score 0, no production of blue staining); low (score 1, time > 20 min), medium (score 2, time 10-20 min) and high (score 3, time < 10 min). The results are shown in Table 9.

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain showed ability to produce H₂O₂. In particular, the strain was classified as a high producer of H₂O₂ (score 3) because the time required for the appearance of blue staining was less than 10 minutes.

Table 9	
Activity tested	DSM 33960
Production of H ₂ O ₂	3

3.3. Production of organic acids and lactic acid

The abilities of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to produce organic acids and lactic acid were quantitatively evaluated by HPLC chromatography following the protocol suggested by Chenoll et al. The test was performed in triplicate, and the results, expressed as mean (mmol/L) and standard deviation, are shown in Table 10.

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain showed ability to produce acetic acid, butyric acid, lactic acid, propionic acid, and succinic acid in high amounts.

Table 10		
Activity tested		DSM 33960
Production of organic acids and lactic acid (mmol/L)	Acetic acid	18.31±1.13
	Butyric acid	39.61±2.18
	Lactic acid	799.29±2.21
	Propionic acid	98.54±1.06
	Succinic acid	98.48±1.83

3.4. Anti-inflammatory activity in macrophages

U937 cell lines (human pro-monocytic cell line), were used as an *in vitro* model of inflammation. U937 cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/L D-glucose (Gibco, Life Technologies, Milan, Italy) supplemented with 10% v/v bovine serum (FBS) (Invitrogen, Carlsbad, California, USA), 1% penicillin/streptomycin (Carlo Erba, Milan, Italy) and 60 mg/mL gentamicin (Gibco). U937 cells were differentiated into macrophages by treatment with PMA 200 nM (Phorbol 12-myristate 13-acetate) for 72 h. In order to simulate the inflammatory process, the cells were pretreated with lipopolysaccharide (LPS) at a concentration of 100 ng/mL for 2 h. The anti-inflammatory effect of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was evaluated by treating differentiated cells with the strain under study at a concentration of 10 µg/mL for 6 hours. At the end of treatment, cells were washed with PBS, harvested by trypsinization and then lysed for RNA extraction. Quantification of COX-1 (Cyclooxygenase-1), COX-2, IL-8 and IL-10 genes was performed by real-time qRT-PCR. Untreated cells were used as controls. The analyses were performed in triplicate, and the results are shown in Table 11 as mean and standard deviation.

The analysis shows that *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain exhibits anti-inflammatory activity, resulting in the reduction of gene expression of COX-2 and pro-inflammatory interleukin IL-8 and increase of anti-inflammatory interleukin IL-10 (Table 11).

Table 11		
Activity tested		DSM 33960
Anti-inflammatory activity	COX-1 (LPS 2.13 ± 0.11)	$2,19 \pm 0,26$
	COX-2 (LPS 15.29 ± 0.73)	$0,09 \pm 0,07$
	IL-8 (LPS 4.16 ± 0.05)	$0,71 \pm 0,08$
	IL-10 (LPS 128.90 ± 1.28)	$524,71 \pm 1,05$

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3.5. Antioxidant activity

Lactcaseibacillus rhamnosus CA15 DSM 33960 strain was tested for antioxidant activity by ABTS assay using the substrate 2,2-azino di-(3-ethylbenzthiazoline sulfonate). A standard curve was obtained using Trolox at different concentrations (20-1000 M). The results were expressed as molar concentration (M) of Trolox equivalent per liter (mol TE/L). In addition, antioxidant activity was estimated by testing the oxidation of oleic acid. In detail, 1 mL of cell-free supernatant was added to 1 mL of PBS (0.1 M, pH 7.0) and 1 mL of linoleic acid (50 mM) in ethanol (99.5%). Oxidation was measured by ferric thiocyanate determination. Butylated hydroxytoluene (BHT) and α -tocopherol (1 mg/mL) were used as positive controls. Uninoculated MRS broth without antioxidants was used as a negative control. Analyses were performed in triplicate.

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The results of antioxidant activity are shown in Figure 2, and are expressed as absorbance values at 500nm . The tested strain showed antioxidant activity, counteracting linoleic acid peroxidation. The antioxidant activity of *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain was similar ($P < 0.05$) to that found for α -tocopherol.

3.6. Adhesion to cell lines

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The adhesion ability of *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain was tested using intestinal cell lines, Caco-2 (ATCC HTB-37) and HT29 (Caucasian colon adenocarcinoma grade II, Merck, Milan, Italy) and human vaginal epithelial cell lines (VK2/E6E7 ATCC-CRL-2616). Each test was repeated three times in three separate experiments, and the results, shown in Table 12, are expressed as mean percent adhesion (%) and standard deviation. Adhesion ability was expressed as a percentage and was calculated by comparing the number of viable cells tested (8 log ufc/ml) and the number of adherent cells in the cell lines.

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Lactcaseibacillus rhamnosus CA15 DSM 33960 strain demonstrates adhesion ability to all cell lines tested.

Table 12		
Activity tested		DSM 33960
Adhesion to cell lines (%)	Caco-2	36.15 ± 0.32
	HT29	23.39 ± 0.90
	VK2/E6E7	34.71 ± 0.09

3.7. Adhesion to intestinal mucus

The strain under study was labeled by addition to the growth medium (MRS broth) of 10 µl/ml tritiated thymidine (5-3Hthymidine, 120 Ci mmol⁻¹; Amersham Biosciences, Little Chalfont, UK). After incubation at 37 °C for 18 h, cells were washed with PBS and standardized to an OD₆₀₀ of 0.25±0.05 corresponding to a cell number of about 8 log ufc/ml.

A solution of intestinal mucus was prepared by dissolving mucin (Mucin from porcine stomach type III, Merck, Milan, Italy) in NaOH 1M and diluting the solution in 1X PBS at a ratio of 1:20. 150 µl of the intestinal mucus solution was immobilized in wells of polystyrene microplates (Maxisorp, Nunc, Denmark) by overnight incubation at 4 °C. The plates were washed three times with PBS. 100 µl of the radiolabeled and standardized bacterial suspension was transferred to the wells and incubated for 1 h at 37 °C. Subsequently, the wells were washed three times with 200 µl of PBS to remove nonadherent cells. The adherent cells were released and lysed with 1% (w/v) sodium dodecyl sulfate (SDS) in NaOH (0.1 mol/liter) (200 µl per well) by incubation at 37°C overnight. The contents of the wells were transferred to microcentrifuge tubes containing scintillation liquid (OptiPhase "HiSafe 3", Wallac, Turku, Finland), and radioactivity was measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion with respect to the radioactivity of the bacterial suspension added to the immobilized mucus. Adhesion was determined in three independent experiments, and each test was performed in six replicates to calculate intra-test variation.

The results of the ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to adhere to human intestinal mucus are shown in Figure 3. *Lactocaseibacillus rhamnosus* LGG strain was used as the reference strain. In detail, adhesion percentage of 11.99±0.27 and 11.61±0.76 was obtained for CA15 and LGG strains, respectively.

3.8. Biofilm formation

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain was tested for the ability to produce biofilm according to the following. For the test, 96-well polystyrene microplates were used. Cell culture standardized to 9 log ufc/ml was inoculated in 5% (v/v) MRS broth and in MRS broth supplemented with TWEEN 80 (1% v/v) and incubated at 37°C for 24 and 72 hours. After incubation, the wells were washed three times with PBS (pH 7.0) and treated with 200 µl of crystal violet (0.1% w/v), prepared in isopropanol-methanol-PBS solution (1:1:18 v/v), for 30 min. Excess dye was washed with sterile water. After drying for 2-3 hours, the biofilm produced by the strain was quantified by spectrophotometric reading at 595 nm, after treatment of the wells with 200 µL of 30% glacial acetic acid. Optical density (OD_{595nm}) was measured using a microplate absorbance reader iMark™ (Biorad). Sterile medium was used as the negative control (ODc). Biofilm production ability was reported as follows: no biofilm production (OD

$\leq \text{ODc}$); weak biofilm production ($\text{ODc} < \text{OD} \leq 2\text{ODc}$); medium biofilm production ($2\text{ODc} < \text{OD} \leq 4\text{ODc}$); high biofilm production ($4\text{ODc} < \text{OD} \leq 8\text{ODc}$); very high biofilm production ($8\text{ODc} < \text{OD}$). The test was carried out in triplicate and the results are reported in Table 13.

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain showed high ability to produce biofilm in MRS after 24 and 72 h incubation. The ability to produce biofilm was found to be very high after growth for 72 hours in MRS with Tween 80.

Table 13			
Activity tested			DSM 33960
Biofilm production	MRS	24h	High
		72h	High
	MRS + TWIN 80	24h	High
		72h	Very high

3.9. Ability to inhibit the adhesion of *Candida* spp.

- The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to counteract the adhesion of *C. albicans* ATCC 10231, *C. glabrata* ATCC 90030, *C. krusei* ATCC 14243, *C. parapsilosis* ATCC 90018, and *C. tropicalis* ATCC 13803 was evaluated, using 96-well polystyrene microplates, by "pre-coating" and "co-incubation" methods. In both methods, CFS ("cell-free supernatant") of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was obtained by employing a standardized cell culture at 9 log ufc/ml subjected to centrifugation and filtration of the supernatant through 0.22 μm filters.

- Pre-coating: CFS (200 μL) was transferred to wells of polystyrene microplates and incubated at 37 °C for 24 h. After incubation, CFS was removed and the wells were washed twice with 100 μL of PBS (pH 7.2). The cell suspension of each *Candida* species tested (150 μL), standardized to 7 log ufc/ml, was added, in separate wells, and incubated at 37°C for 24 hours. After incubation, the wells were washed gently with PBS (pH 7.2) then 99% methanol (100 μL) was added to fix the biofilm. After 15 minutes, the residual methanol was removed by washing then 2% crystal violet (100 μL) was added. After 20 min, the excess dye was removed and 100 μL of 33% glacial acetic acid was added. Optical density (ODc) was measured at 595 nm, using a microplate absorbance reader iMark™ (Biorad). *Candida* spp. suspension, without CFS, was used as a negative control (OD0). The percentage of *Candida* spp. adhesion in the presence of CFS of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was calculated as follows:

$$\text{adhesion \%} = 1 - \text{ODc} / \text{OD0} \times 100$$

- Co-incubation: 100 μL of CFS of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain and 150 μL of cell suspension of each *Candida* species tested, standardized to 7 log ufc/ml, were transferred to 96-well polystyrene microplates and incubated at 37 °C for 24 h. The washing and staining steps were performed as reported for the

pre-coating test. *Candida* spp. suspension without CFS of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was used as a negative control. The optical density was measured at 650 nm. The percentage of reduction in biofilm formation was calculated as follows:

$$\text{reduction \%} = 1 - \text{OD}_A / \text{OD}_B \times 100$$

indicating with OD_A the optical density of the sample (CFS of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain and cell suspension of each *Candida* species) and with OD_B the optical density of the negative control.

The results for the ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to counteract *Candida* spp. adhesion are reported in Table 14 as the mean and standard deviation of three determinations.

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain is found to inhibit the adhesion of all *Candida* species tested by exhibiting inhibition percentages above 50% in pre-coating assays and above 20% in co-incubation assays.

Table 14			
Activity tested		DSM 33960	
		Pre-coating	Co-incubation
Ability to inhibit the adhesion of <i>Candida</i> spp.	<i>Candida albicans</i> ATCC10231	84.6 ± 0.14	20.1 ± 0.70
	<i>Candida tropicalis</i> ATCC 13803	71.6 ± 0.09	48.4 ± 0.60
	<i>Candida glabrata</i> ATCC 90030	87.0 ± 0.2	29.6 ± 0.39
	<i>Candida krusei</i> ATCC 14243	50.1 ± 0.17	45.9 ± 0.70
	<i>Candida parapsilosis</i> ATCC 90018	75.7 ± 0.2	23.9 ± 0.88

3.10. Inhibition of pathogen adhesion

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to inhibit pathogen adhesion was tested in polystyrene plates by transfer ring, to each well, 100 µl of a cell suspension of the strain standardized to 8 log cfu/ml. After incubation for 1 hour at 37°C, unbound cells were removed by washing twice with PBS buffer. The tested pathogen strains were previously grown in liquid medium and radiolabeled. 100 µl of each pathogen, standardized to 8 log cfu/ml, was added to the wells and incubated at 37°C for 1 hour. Then, unbound labeled bacteria were removed by washing, and bound bacterial cells were released and lysed with 1% (w/v) SDS in NaOH 0.1 M. After incubation at 37°C overnight, radioactivity was assessed by liquid scintillation. The results are shown in Table 15 and are expressed as percentage of radioactivity recovered after adhesion, compared with the radioactivity of the bacterial suspension added to the wells. The ability to inhibit pathogen adhesion was expressed as a percentage and calculated as the difference between pathogen adhesion in the absence and presence of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain. Inhibition was determined in three independent experiments, and each test was performed in six replicates.

Table 15	
Pathogenic strains	DSM 33960
<i>Streptococcus agalactiae</i> DSM 2134	87.41 ± 0.08
<i>Enterobacter cloacae</i> DSM 30054	82.00 ± 0.26
<i>Staphylococcus aureus</i> DSM 1104	81.58 ± 0.15
<i>Candida glabrata</i> ATCC 90030	53.44 ± 0.40
<i>Pseudomonas aeruginosa</i> DSM 1117	71.15 ± 0.19
<i>Enterococcus faecalis</i> DSM 2570	62.68 ± 0.34
<i>Gardnerella vaginalis</i> DSM 14018	67.43 ± 0.08
<i>Candida parapsilosis</i> ATCC 90018	86.24 ± 0.15
<i>Candida albicans</i> ATCC10231	91.53 ± 1.19

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain showed ability to inhibit adhesion of all tested pathogens with inhibition percentages above 50%.

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3.11. Inhibition of the development of adherent pathogens

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to counteract previously adhered pathogens was assayed as follows. In detail, radiolabeled and standardized pathogens, as reported previously, were added to the wells and incubated for 1 h at 37°C. Unbound pathogens were removed by washing three times with PBS buffer, and the nonradiolabeled *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain (100 µl, 8 log cfu/ml) was added to each well. The plates were incubated for 1 h at 37°C. Then, the wells were washed again and the bound bacteria were released and lysed as described above. Radioactivity was measured as described above, and the results obtained are shown in Table 16. The ability to counteract previously adhered pathogens was calculated as the difference between pathogen adhesion before and after the addition of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain. Three independent experiments were performed. Each test was performed in six replicates to calculate intra-test variation.

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Table 16	
Pathogenic strains	DSM 33960
<i>Streptococcus agalactiae</i> DSM 2134	73.11 ± 0.16
<i>Enterobacter cloacae</i> DSM 30054	52.00 ± 0.14
<i>Staphylococcus aureus</i> DSM 1104	51.56 ± 0.11
<i>Candida glabrata</i> ATCC 90030	92.79 ± 0.14
<i>Pseudomonas aeruginosa</i> DSM 1117	62.12 ± 0.21
<i>Enterococcus faecalis</i> DSM 2570	52.33 ± 0.24

<i>Gardnerella vaginalis</i> DSM 14018	54.86 ± 0.56
<i>Candida parapsilosis</i> ATCC 90018	77.07 ± 0.19
<i>Candida albicans</i> ATCC10231	63.27 ± 1.02

Lactocaseibacillus rhamnosus CA15 DSM 33960 strain showed ability to inhibit all tested pathogens with percentages above 50%.

5 3.12. Production of exopolysaccharides (EPS)

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to produce exopolysaccharides (EPS) was evaluated using the phenol/sulfuric acid method, and the amount of EPS produced was assessed using glucose (50-500 mg/l) as a standard.

The assay was performed in triplicate, and the results expressed in mg/L are shown in Table 17.

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Table 17	
Activity tested	DSM 33960
EPS Production (mg/L)	292 ± 2.13

3.13. Hydrophobicity, auto-aggregation and co-aggregation

Hydrophobicity (H%) was determined by mixing equal volume of xylene and cell suspension, of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, in PBS (9 log cfu/ml). After incubation at 37° C for 10 min, the suspension was kept at 37° C for 5 h to achieve phase separation. The aqueous phase was taken and subjected to absorbance determination at 600 nm. Hydrophobicity was calculated as the percentage reduction in absorbance ($\Delta\text{Abs} \times 100$) of the aqueous phase after incubation compared with the absorbance of the base suspension according to the following formula:

$$H\% = \Delta\text{Abs}/\text{Abs}_{10} \times 100.$$

20 Auto-aggregation (Auto-A%) was determined by using 1ml of cell suspension (9 log ufc/ml), of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, in PBS buffer, vortexed for 10 seconds and incubated at room temperature for 5 hours. Then, an aliquot (0.1 ml) of the upper phase of the suspension was mixed with 500 µl of PBS buffer and the absorbance at 600 nm was determined. Auto-aggregation (Auto-A%) was expressed as the percentage reduction in absorbance after 5 h ($\Delta\text{Abs} \times 100$) compared with that of the original suspension (Abs_0) as follows:

$$25 \quad \text{Auto-A\%} = \Delta\text{Abs}/\text{Abs}_0 \times 100$$

Co-aggregation (Co-A%) was evaluated using *E. coli* 555, *G. vaginalis* ATCC 14018, *G. vaginalis* ATCC 14019, *C. albicans* ATCC 10231, *C. glabrata* ATCC 90030, *C. krusei* ATCC 14243, *C. parapsilosis* ATCC 90018, and *C. tropicalis* ATCC 13803 strains as co-aggregation partners. In detail, equal volume (2 mL) of the cell suspension of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain and each of the pathogenic strains were mixed and vortexed for 30 seconds then kept at room temperature for 5 h. The absorbance at 600nm of the DSM

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33960+pathogen mixture ($Abs_{(CA15+pat)}$) and DSM 33960 strain alone (Abs_{CA15}) was determined. Co-aggregation was calculated as follows:

$$Co-A\% = ((Abs_{CA15} + Abs_{pat})/2 - Abs_{(CA15+pat)}) / (Abs_{CA15} + Abs_{pat}/2) \times 100.$$

The results are reported in Table 18 as the mean and standard deviation of three determinations.

- 5 *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain exhibited good auto-aggregation, hydrophobicity, and co-aggregation with all pathogens tested, indirectly evidencing the ability to adhere to the intestinal mucosa and prevent pathogen development.

Table 18		
Activity tested		DSM 33960
H%		86.13±0.05
Auto-A%		71.80±0.07
Co-A %	<i>E. coli</i> 555	64.18±0.16
	<i>G. vaginalis</i> ATCC 14018	71.23±0.11
	<i>G. vaginalis</i> ATCC 14019	68.18±0.33
	<i>C. albicans</i> ATCC10231	86.12±0.17
	<i>C. glabrata</i> ATCC 90030	82.63±0.18
	<i>C. krusei</i> ATCC 14243	85.14±0.11
	<i>C. parapsilosis</i> ATCC 90018	79.21±0.21
	<i>C. tropicalis</i> ATCC 13803	73.15±0.13

10 3.14. Lysozyme resistance

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to survive in the presence of lysozyme (100mg/L) was evaluated in MRS broth after 0, 30, and 120 min of incubation at 37°C. Lysozyme tolerance was determined by in-plate counting of viable cells. The ability to survive in the presence of lysozyme was assessed by determination of the survival rate (SR%), that is the survival percentage, which was calculated by taking into

- 15 account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100). The value 80% was considered as the minimum survival limit. Cell cultures, of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, in the absence of lysozyme were used as control. The analysis was performed in triplicate.

The results, expressed as mean survival rate (SR%), are shown in Table 19.

The tested strain showed ability to survive in the presence of lysozyme, presenting survival % of 95% and 93 %

- 20 after incubation at 37°C for 30 and 120 min, respectively.

Table 19		
Activity tested		DSM 33960
Lysozyme resistance	30 min	95%
	120 min	93%

3.15. Tolerance to low pH values

The ability of *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain to tolerate low pH values was tested in MRS broth acidified to pH 2.0 and pH 3.0 by addition of HCl 1 M. MRS broth at pH 6.2 was used as a control. The strain under study was revitalized in MRS broth, and the cell suspension in exponential growth phase of growth (9 log ufc/ml) was inoculated into the acidified culture medium. Aliquots were taken immediately after inoculation (0 h) and after 2 h and 4 h of incubation at 37°C. Tolerance to low pH values was determined by in-plate counting of viable cells. The percentage (%) of survival, calculated by taking into account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100) was evaluated, and the value 80% was considered as the minimum survival limit.

The results are shown in Table 20 and expressed as survival % (SR%).

The tested strain showed ability to survive at pH 3.0 and 2.0, presenting survival % greater than 80% after incubation at 37°C for 2 hours and 4 hours (Table 20). The survival percentage at pH 3.0 after 4 hours was 86%. The survival percentage at pH 2.0 after 4 hours is 83%.

Table 20		
Activity tested		DSM 33960
Resistance to pH 3.0	2h	92%
	4h	86%
Resistance to pH 2.0	2h	89%
	4h	83%

3.16. Tolerance to bile salts

The ability of *Lacticaseibacillus rhamnosus* CA15 DSM 33960 strain to survive in the presence of bile salts (bovine bile salts, Oxgall; Sigma-Aldrich), at final concentrations of 0.5% and 1.0% was evaluated as follows. The strain under study was revitalized in MRS broth, and the cell suspension in exponential growth phase (9 log ufc/ml) was inoculated into MRS broth containing the above percentages of bile salts. MRS without bile salts was used as a control. Aliquots were taken immediately after inoculation (0 h) and after 2 h and 4 h of incubation at 37 °C, and viable cells were counted in plates. The analysis was carried out in triplicate and the results, shown in Table 21, are expressed as the survival percentage (SR%), calculated by taking into account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100) and the value 80% was considered as the minimum survival limit.

The tested strain showed ability to survive in the presence of bile salts at the concentrations of 0.5% and 1%. The survival rate to 0.5% bile salts after 4 hours of incubation is 87%. The survival rate to 1% bile salts after 4 hours was 82%.

Table 21		
Activity tested		DSM 33960
Bile 0.5%	2h	91%
	4h	87%
Bile 1%	2h	89%
	4h	82%

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3.17. Survival during simulation of gastrointestinal (GI) digestion

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to survive during gastrointestinal (GI) transit was determined *in vitro* by using both a static system (SGJ/SIF) and a dynamic system (SHIME), the latter capable of exactly reproducing the physiological, chemical and microbiological conditions of the GI tract *in vitro*.

10 3.17-A. Survival during simulation of gastrointestinal (GI) digestion by SGJ/SIF system

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to survive during gastrointestinal (GI) transit was determined *in vitro* using simulated gastric juice ("SGJ") and simulated intestinal fluid ("SIF"). In detail, SGJ (0.3% pepsin, 0.5% NaCl, adjusted to pH 2 by adding HCl 1 M) and SIF (0.1% pancreatin, 0.5% bile salt, 0.5% NaCl, 0.4% phenol, adjusted to pH 8 by adding NaOH 1 M) were prepared immediately before use and sterilized using a 0.22 µm cellulose acetate filter (Minisart filters, Sartorius, Göttingen, Germany). All chemicals were obtained from Sigma Aldrich (St. Louis, MO). Cell cultures in exponential growth phase (9 log cfu/ml) were pelleted by centrifugation and resuspended in phosphate buffered saline (PBS). The resulting cell suspension was mixed with SGJ and incubated for 2 h at 37 °C under stirring (200 rpm). The cells, pelleted by centrifugation, were again suspended in SIF and incubated at 37°C for 3 hours. SGJ and SGJ-SIF-treated cells were subjected to in-plate counting of viable cells. The analysis was performed in triplicate and the results, shown in Table 19, are expressed as survival %.

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The strain exhibited excellent survival during simulated gastrointestinal transit under static conditions. During simulated gastrointestinal digestion, survival greater than 90% was observed. In detail SR% of 99.7% was found after treatment with simulated gastric juice (SGJ) and SR% of 95.7% was found after treatment with simulated intestinal fluid (SIF) (Table 22).

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Table 22		
Activity tested		DSM 33960
Survival during simulation of GI digestion	Simulated gastric juice (SGJ)	99.7%
	Simulated Intestinal Fluid (SIF)	95.7%

3.17-B. Survival during gastrointestinal transit using the dynamic SHIME® system under fasting and full stomach conditions

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to survive during gastrointestinal (GI) transit, both under fasting and full stomach conditions, was determined by using the *Simulator of the Human Intestinal Microbial Ecosystem* (SHIME®, ProDigest, Belgium). This system is capable of accurately simulating the entire human GI tract, incorporating stomach, small intestine and different regions of the colon. Since it is a dynamic system, the physiological, chemical and microbiological conditions of the GI tract are exactly reproduced.

The dynamic SHIME system was set up by using a double-walled reactor, simulating the entire GI tract, maintained at a temperature of 37°C, under constant stirring and anaerobic conditions by nitrogen insufflation. *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was tested in capsule form with a population density of 10 log cfu/g and maintained in the SHIME system within a specific capsule holder.

Survival under fasting conditions

The stomach microenvironment was reproduced by employing gastric juice at pH 2 consisting of: KCl 0.66 g/L, NaCl 3.63 g/L, mucin 3.95 g/L, lecithin 3.4 g/L and pepsin 10 g/L. Digestion at the stomach level was simulated for 45 min. Next, the gastric digest was subjected to intestinal digestion then pancreatic juice consisting of: NaHCO₃ 2.6 g/L, bile salts 4.8 g/L, pancreatin 1.9 g/L, trypsin 10 g/L and chymotrypsin 10 g/L was added. Therefore, duodenal (27 min incubation with controlled pH increase from 2.0 to 6.5), jejunal (63 min incubation with controlled pH increase from 6.5 to 7.5) and ileal (90 min incubation at constant pH of 7.5) fractions were simulated. During intestinal digestion, aliquots of NaHCO₃ (8.4 g/L) were added after 60, 90 and 120 min to determine the pH increase to the above values. The pH was continuously monitored using Senseline F410 pH meter (ProSense, Oosterhout, The Netherlands). Solutions of HCl (0.5 M) and NaOH (0.5 M) were dosed automatically, using a pump system, in order to determine a constant maintenance of pH within the above ranges. In parallel, a negative control (digestion as above but in the absence of the encapsulated *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain) was set up to exclude the presence of contamination as well as to monitor process parameters. All assays were performed in duplicate.

In order to determine the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain during GI transit, samples of gastric, duodenal, jejunal, and ileal digests were taken sterilely and subjected to culture-dependent analysis. In detail, 5 ml of sample were subjected to serial dilutions employing anaerobic PBS (K₂HPO₄, 8.8 g/L; KH₂PO₄, 6.4 g/L; NaCl, 8.5 g/L; L-cysteine HCl, 0.5 g/L) and the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was determined by in-plate counting of viable cells employing Lactobacilli MRS agar medium

(BD Difco™) after incubation in anaerobiosis at 37°C for 48 h. The percentage (%) of survival was evaluated, calculated by taking into account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100). The percentage of capsule degradation was determined visually.

Table 23 shows the results for the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, expressed as log cfu/ml and survival % (SR%), as well as the capsule degradation rate.

In detail, taking into account the initial population density of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain of 10 log cfu/g, SR% values greater than 50% were found in the duodenum, jejunum and ileum demonstrating high ability to survive the stressful conditions of the GI tract. Given the extremely acidic conditions of the stomach (pH 2) as well as the presence of pepsin, the low presence of viable cells in the stomach could be attributable to a viable and non-culturable condition of the strain. With reference to the degradation percentage of the capsule, it was found that, the capsule gradually dissolves in the various tracts of the GI system, reaching a degradation percentage of 100% in the jejunum.

Table 23				
Activity tested		DSM 33960		
		Log ufc/ml	SR%	% of capsule degradation
<i>Survival during gastrointestinal transit under fasting conditions</i>	Stomach	< 1	<1.0%	50%
	Duodenum	7.10±0.6	66.9%	80%
	Jejunum	7.39±0.01	69.7%	100%
	Ileum	7.35±0.6	69.3%	100%

15 *Survival under full stomach conditions*

The stomach microenvironment was reproduced using gastric juice at pH 4.5 containing SHIME® nutrient medium (PDNM001B 20.53 g/L, ProDigest, Ghent, Belgium), NaCl 3.63 g/L, KCl 0.65 g/L, lecithin 13.5 g/L and pepsin 40 g/L. Digestion at the stomach level was simulated for 120 min. Next, the gastric digest was subjected to intestinal digestion then pancreatic juice consisting of: NaHCO₃ 7.7 g/L, bile salts 15 g/L, pancreatin 10 g/L, trypsin 10 g/L and chymotrypsin 10 g/L was added. Therefore, duodenal (27 min incubation with controlled pH increase from 2.0 to 6.5), jejunal (63 min incubation with controlled pH increase from 6.5 to 7.5) and ileal (90 min incubation at constant pH of 7.5) fractions were simulated. During intestinal digestion, aliquots of NaHCO₃ (8.4 g/L) were added after 60, 90 and 120 min to determine the pH increase to the above values. The pH was continuously monitored using Senseline F410 pH meter (ProSense, Oosterhout, The Netherlands). Solutions of HCl (0.5 M) and NaOH (0.5 M) were dosed automatically, using a pump system, in order for the pH to be constantly maintained within the above ranges. In parallel, a negative control was set up (digestion as above but in the absence of the encapsulated *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain), aimed at excluding the presence of contamination as well as monitoring process parameters. All assays were performed in duplicate.

In order to determine the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain during GI transit, samples of digests were taken sterilely in the end stages of the stomach, duodenum, jejunum, and ileum and subjected to culture-dependent analysis. In detail, 5 ml of sample were subjected to serial dilutions employing anaerobic PBS (K_2HPO_4 , 8.8 g/L; KH_2PO_4 , 6.4 g/L; NaCl, 8.5 g/L; L-cysteine HCl, 0.5 g/L) and the survival of

5 *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was determined by in-plate counting of viable cells employing Lactobacilli MRS agar medium (BD Difco™) after incubation in anaerobiosis at 37°C for 48 h. The percentage (%) of survival was evaluated, calculated by taking into account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100).

The results for survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, expressed as log cfu/ml and survival % (SR%), as well as the percentage of capsule degradation are shown in Table 24.

In detail, taking into account the initial population density of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain, which was 10 log ufc/g, the strain showed SR% values above 50% in all the GI system fractions tested. With reference to the capsule degradation rate, it was found that, the capsule, completely disintegrates (100% degradation percentage) in the duodenum.

15

Table 24				
Activity tested		DSM 33960		
		Log ufc/ml	SR%	% of capsule degradation
Survival during gastrointestinal transit under full stomach conditions	Stomach	6.27±0.03	59.2%	70%
	Duodenum	6.70±0.1	63.2%	100%
	Jejunum	6.79±0.3	64.0%	100%
	Ileum	6.03±0.03	56.9%	100%

3.17-C. Survival during gastrointestinal transit using the dynamic SHIME system under fasting conditions and in the presence of antibiotic (fluconazole 200mg)

The ability of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain to survive during gastrointestinal (GI) transit, under fasting conditions and in the presence of the antibiotic fluconazole (200mg), was determined by employing

20 the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®, ProDigest, Belgium). The dynamic SHIME system was set up by employing a double-walled reactor, simulating the entire GI tract, maintained at a temperature of 37°C, under constant stirring and anaerobic conditions by nitrogen insufflation. *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain and the antibiotic were introduced simultaneously inside the reactor. In

25 detail, *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was tested in capsule form with a population density of 10 log cfu/g and maintained in the SHIME system within a specific capsule holder. The antibiotic fluconazole (200mg), previously dissolved in 50 ml of sterile water as per the dosage indication, was introduced sterilely through additional port.

- The stomach microenvironment was reproduced by employing gastric juice at pH 2 consisting of: KCl 0.66 g/L, NaCl 3.63 g/L, mucin 3.95 g/L, lecithin 3.4 g/L and pepsin 10 g/L. Digestion at the stomach level was simulated for 45 min. Next, the gastric digest was subjected to intestinal digestion then pancreatic juice consisting of: NaHCO₃ 2.6 g/L, bile salts 4.8 g/L, pancreatin 1.9 g/L, trypsin 10 g/L and chymotrypsin 10 g/L was added.
- 5 Therefore, duodenal (27 min incubation with controlled pH increase from 2.0 to 6.5), jejunal (63 min incubation with controlled pH increase from 6.5 to 7.5) and ileal (90 min incubation at constant pH of 7.5) fractions were simulated. During the intestinal phase, after 60, 90 and 120 min, 8.4 g/L of NaHCO₃ were added to determine the pH increase to the above values. The pH was continuously monitored using Senseline F410 pH meter (ProSense, Oosterhout, The Netherlands). Solutions of HCl (0.5 M) and NaOH (0.5 M) were dosed automatically,
- 10 using a pump system, in order for the pH to be constantly maintained within the above ranges. In parallel, a negative control was set up with antibiotic only and digestion was conducted as above but in the absence of the encapsulated *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain. This control is aimed at excluding the presence of contamination as well as monitoring process parameters. All assays were performed in duplicate.
- In order to determine the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain in the presence of
- 15 fluconazole, digest samples were taken sterically in the end stages of the stomach, duodenum, jejunum, and ileum and subjected to culture-dependent analysis. In detail, 5 ml of sample were subjected to serial dilutions employing anaerobic PBS (K₂HPO₄, 8.8 g/L; KH₂PO₄, 6.4 g/L; NaCl, 8.5 g/L; L-cysteine HCl, 0.5 g/L) and the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain was determined by in-plate counting of viable cells employing Lactobacilli MRS agar medium (BD Difco™) after incubation in anaerobiosis at 37°C for 48 h.
- 20 The percentage (%) of survival was evaluated, calculated by taking into account the final (cfuF) and initial (cfuI) population density (cfuF/cfuI *100). The percentage of capsule degradation was determined visually.
- The results for the survival of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain in the presence of fluconazole (200mg), expressed as log cfu/ml and survival % (SR%), as well as the percentage of degradation of capsule of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain are shown in Table 25.
- 25 In detail, taking into account the initial population density of *Lactocaseibacillus rhamnosus* CA15 DSM 33960 strain of 10 log cfu/g, the strain showed SR% values above 60% in the duodenum, jejunum and ileum demonstrating high ability to survive the stressful conditions of the GI tract. Given the extremely acidic conditions of the stomach (pH 2) as well as the presence of pepsin, the absence of viable cells in the stomach could be attributable to a viable but non-culturable condition of the strain. With reference to the degradation percentage of the capsule, it
- 30 was found that, the capsule gradually dissolves in the various tracts of the GI system, reaching a degradation percentage of 100% in the jejunum.

Table 25				
Activity tested		DSM 33960		
		Log ufc/ml	SR%	% of capsule degradation
<i>Survival during gastrointestinal transit under fasting conditions and in the presence of fluconazole (200mg)</i>	Stomach	<1	<1.0%	50%
	Duodenum	7.12±0.4	67.1%	80%
	Jejunum	7.10±0.2	67.0%	100%
	Ileum	6.96±0.1	65.7%	100%

3.18. Acidifying capacity in MRS medium

The acidifying capacity of *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain was tested in de Man Rogosa and Sharp broth medium (MRS, Oxoid, Milan, Italy) and monitored for 24 h by pH determination. In detail, an overnight culture of *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain, at a population density of 8.60 cfu/ml, was transferred into 30 ml of MRS broth medium and incubated, under constant stirring, at a temperature of 37°C. The pH of the medium was determined at regular time intervals by pH meter (XS, PH 60 VIO LAB).

Table 26 shows the pH values measured at different sampling times.

In detail, *Lactcaseibacillus rhamnosus* CA15 DSM 33960 strain resulted in a gradual decrease in the pH of the medium used, reaching pH values of 3.82 after 24 hours of incubation at 37°C.

Table 26		
Activity tested		DSM 33960
<i>Acidifying activity</i>	Time (h)	pH
	0	6.2
	2	5.92
	3	5.3
	5	5.26
	8	4.89
	15	4.43
	16	4.25
	18	4
	20	3.92
	24	3.82

CLAIMS

1. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited at the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* -DSMZ, with deposit number DSM 33960.
2. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, according to claim 1, for use as a medicament.
3. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, according to claim 1 or 2, for use in a method for the prevention and/or treatment of diseases related to dysbiosis of the microbiota of the urogenital tract, preferably the female urogenital tract.
4. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to claim 3 , wherein said dysbiosis refers to dysbiosis of the vaginal microbiota.
5. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to claim 3 or 4, wherein said diseases related to dysbiosis of the microbiota of the female urogenital tract are selected from the group comprising or alternatively consisting of vaginal infections, urogenital tract infections, vaginal inflammation, inflammation of the urogenital tract, bacterial vaginosis (BV) and recurrences thereof, vulvovaginal candidiasis (VVC), sexually transmitted bacterial infections preferably carried by *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis*, mixed dysbiosis, group B *Streptococci* (GBS) infections.
6. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to claim 5, wherein said urogenital tract infections are selected from infections/inflammations due to one or more microorganisms such as *Gardnerella vaginalis*, *Escherichia coli*, *Staphylococcus aureus*, *Candida* spp, *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, and *Streptococcus agalactiae* and/or combinations thereof.
7. *Lactiseibacillus rhamnosus* CA15 bacterial strain , deposited under DSM deposit number 33960, for use according to any one of claims 1 to 6, wherein said bacterial strain is administered orally.
8. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to any one of claims 1 to 7, wherein said bacterial strain is in lyophilized form.

9. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to any one of claims 1 to 8, wherein said bacterial strain is administered in combination with one or more antimicrobial agents.
- 5 10. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to claim 9, wherein said one or more antimicrobial agents are selected from metronidazole, clotrimazole, boric acid, fluconazole and mixtures thereof.
- 10 11. *Lactiseibacillus rhamnosus* CA15 bacterial strain, deposited under DSM deposit number 33960, for use according to any one of claims 1 to 9, wherein said bacterial strain is administered in capsule form.