

“PROBIOTICS FOR USE IN DIVERTICULOSIS AND DIVERTICULAR DISEASE”

DESCRIPTION

The present invention relates to the use of probiotics to control the inflammatory process of the colon in subjects with structural modifications of the colon wall, preferably in subjects with diverticulosis, preferably affected by diverticular disease.

5

DETAILED DESCRIPTION

A first aspect of the present invention relates to the use of probiotics for the treatment and/or prevention and/or improvement of the symptoms associated with diverticulosis, in particular diverticular disease. The diverticular disease is preferably symptomatic uncomplicated diverticular disease (SUDD), or segmental colitis associated with diverticulosis (SCAD). Diverticulosis, associated with symptomatic or asymptomatic diverticular disease, to which in general the present invention makes reference, is associated with an over-expression of NO and/or iNOS and/or at least one pro-inflammatory cytokine, preferably selected from TNF α , IL1 β , IL-6b, IL-10 and combinations thereof. For the purposes of the present invention, the probiotics can be taken as an adjuvant to any therapy designed to treat or prevent diverticulosis and the preferred forms described above, i.e. taking probiotics, preferably on a regular basis as described in greater detail below, shows to favour a positive outcome of the presently available treatments designed to treat and/or prevent this pathology in its different forms. Therefore, the use of probiotics aimed at treating and/or preventing and/or improving the symptoms associated with diverticulosis, in particular, asymptomatic diverticulosis and/or diverticular disease, may be associated or combined with further therapeutic approaches, preferably of a pharmacological or socio-behavioural type, for example a healthy lifestyle or diet.

In this context, the definition of “probiotic” is the one formulated by a group of experts jointly convened in 2001 by the FAO and the WHO: “Live microorganisms which when administered in adequate amounts confer a health benefit on the host”. In particular, in Italy, the Ministry of Health has defined probiotics as “microorganisms which demonstrate to be able, once ingested in sufficient amounts, to exert functions that are beneficial for the body”, substantially echoing the definition of the two above-mentioned organisations.

The probiotics to which the present invention relates are based on bacteria and/or yeast and/or other microorganisms.

According to a preferred aspect of the present invention, the bacteria preferably belong to a genus selected from: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Aerococcus*, *Enterococcus* and combinations thereof. The genus that is preferred for the purposes of the present invention is *Lactobacillus* and/or *Bifidobacterium*.

According to a further preferred aspect of the invention, the bacteria of the genus *Lactobacillus* belong to at least one of the following species: *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus alimentarius*, *Lactobacillus aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus johnsonii*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus collinoides*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus sakei*, *Lactobacillus salivarius* and *Lactobacillus sanfranciscensis*.

Particularly preferred for the purposes of the present invention is the bacterial species *Lactobacillus paracasei*, preferably the strain DG® (*Lactobacillus paracasei* CNCM I-1572).

The bacterial strain L. casei DG® (*Lactobacillus paracasei* CNCM I1572) was deposited by SOFAR S.p.A. with the National Collection of Microorganism Cultures of the Pasteur Institute of Paris on 05/05/1995, with the deposit number CNCM I-1572.

According to a further preferred aspect of the invention, the bacteria of the Bifidobacterium genus belong to at least one of the following species: *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, *B. adolescentis*, *B. catenulatum*, *B. angulatum*, *B. asteroides*, *B. boum*, *B. choerinum*, *B. coryneforme*, *B. cuniculi*, *B. denticolens*, *B. dentium*, *B. gallicum*, *B. gallinarum*, *B. indicum*, *B. inopinatum*, *B. lactis*, *B. magnum*, *B. merycicum*, *B. minimum*, *B. pseudocatenulatum*, *B. pseudolongum*, *B. pullorum*, *B. ruminantium*, *B. saeculare*, *B. subtile*, *B. thermacidophilum*, *B. thermophilum* and *B. tsurumiense*.

According to a further preferred aspect of the invention, the bacteria of the Bacillus genus belong to at least one of the following species: *Bacillus clausii*, *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus megaterium*, *Bacillus halodurans*, [*Bacillus thuringiensis*](#), *Bacillus insolitus* and *Bacillus marinus*.

According to a further preferred aspect of the invention, the bacteria of the Propionibacterium genus belong to at least one of the following species: *P. shermanii*, *P. acnes*, *P. australiense*, *P. avidum*, *P. cyclohexanicum*, *P. freudenreichii*, *P. granulosum*, *P. jensenii*, *P. microaerophilum*, *P. propionicum* and *P. thoenii*.

According to a further preferred aspect of the invention, the bacteria of the Streptococcus genus belong to at least one of the following species: *Streptococcus thermophilus*, *Streptococcus salivarius*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus constellatus*, *Streptococcus downei*, *Streptococcus dysgalactiae*, *Streptococcus equinus*, *Streptococcus ferus*, *Streptococcus*

infantarius, *Streptococcus iniae*, *Streptococcus intermedius*, *Streptococcus milleri*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus orisratti*, *Streptococcus parasanguinis*, *Streptococcus peroris*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus pyogenes*, *Streptococcus ratti*, *Streptococcus tigurinus*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus vestibularis*, *Streptococcus viridans* and *Streptococcus zooepidemicus*.

According to a further preferred aspect of the invention, the bacteria of the *Lactococcus* genus belong to at least one of the following species: *L. chungangensis*, *L. formosensis*, *L. fujiensis*, *L. garvieae*, *L. lactis*, *L. piscium*, *L. plantarum*, *L. raffinolactis* and *L. taiwanensis*

According to a further preferred aspect of the invention, the bacteria of the *Aerococcus* genus belong to at least one of the following species: *A. urinae*, *A. sanguinicola*, *A. christensenii*, *A. suis*, *A. urinaeequi* and *A. urinaehominis*.

According to a further preferred aspect of the invention, the bacteria of the *Enterococcus* genus belong to at least one of the following species: *Enterococcus avium*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus haemoperoxidus*, *Enterococcus hirae*, *Enterococcus malodoratus*, *Enterococcus moraviensis*, *Enterococcus mundtii*, *Enterococcus pseudoavium*, *Enterococcus raffinosus* and *Enterococcus solitarius*.

According to a further preferred aspect of the invention, the yeasts belong preferably to the *Saccharomyces* genus, more preferably to the species *Saccharomyces cerevisiae* and/or *Saccharomyces boulardii*.

A further aspect of the present invention relates to a composition comprising the probiotics as described above.

The microorganisms of the composition of the present invention are

preferably live, and thus the composition is also definable as probiotic. Alternatively, the microorganisms that can be used are dead or tyndallized.

In a further embodiment, the microorganisms are in the form of a lysate or extract and therefore the composition is also definable as a paraprobiotic, or a single component, or several components thereof, present at the level of the bacterial wall.

In a further embodiment of the invention, the composition further comprises the metabolic bioproducts generated by microorganisms, defined as postbiotics, and/or any other product of bacterial derivation. Therefore, the composition of the present invention is also a probiotic or a paraprobiotic or a postbiotic, known or presumed, or a component of the bacterial wall.

In general, the microorganisms comprised in the composition of the present invention are single microorganisms or combinations of any microbial species included in the QPS list of the EFSA.

Preferably the bacteria comprised in the composition are capable of surviving gastrointestinal transit and thus of reaching the colon live.

The microorganisms of the present invention are preferably administered in an amount ranging from 1 billion to 100 billion, more preferably 10-75 billion, even more preferably 15-50 billion, most preferably 20-30 billion cells, preferably bacterial cells, per administration.

According to a preferred aspect, the administration of microorganisms, preferably of bacteria, takes place at least 1-2 times a day.

Every route of administration is envisaged in the present invention. Preferably, the administration of the composition and/or probiotics, preferably based on bacteria, is oral, more preferably in the form of pills, capsules, tablets, granular powder, hard-shelled capsules, orally dissolving granules, sachets, lozenges or drinkable vials.

Alternatively, the composition of the invention and/or the probiotics, preferably based on bacteria, is (are) formulated as a liquid, for example

as a syrup or beverage, or else it is (they are) added to food, for example to a yogurt, cheese or fruit juice. Alternatively, the composition of the invention and/or the probiotics, preferably based on bacteria, is (are) formulated in a form capable of exercising a topical action, for example as an enema.

The oral formulation of the composition and/or probiotics to which the present invention relates, preferably based on bacteria, further comprises excipients generally accepted for the production of probiotic and/or pharmaceutical products.

According to a preferred aspect of the invention, the composition further comprises anti-caking agents, preferably silicon dioxide or magnesium stearate.

According to a preferred aspect of the invention, the composition further comprises coating agents, preferably gelatine.

In a further embodiment of the invention, the composition of the invention comprises vitamins, trace elements, preferably zinc or selenium, enzymes and/or prebiotic substances, preferably fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, guar gum or combinations thereof.

EXAMPLE

The experimental study underlying the present invention is based on biopsy samples isolated from 40 patients. The subjects were thus stratified:

- 10 patients with asymptomatic diverticulosis, 3 of whom female; age: 59-77);
- 10 patients with symptomatic uncomplicated diverticular disease (SUDD), 3 of whom female; age: 44-92);
- 10 patients with the symptomatic uncomplicated diverticular disease preceded by acute diverticulitis, 2 of whom female; age: 31-77);
- 10 patients without gastrointestinal problems, 6 of whom female; age: 33-75).

Six biopsy samples were obtained from the sigmoid colon of each patient;

in the patients with diverticula, the biopsies were isolated in proximity thereto.

Informed consent was obtained from all patients and approval was obtained from the ethics committee of the University of Naples Federico II. The polarized biopsies were placed on a sterilized metal grid with the mucosal side down and the serosal side up. Then the metal grid was placed at the centre of an organ culture dish for 24 hours and maintained in DMEM-F12 at 37°C, in the presence of 95% oxygen and 5% CO₂. The biopsies were then stimulated with the pathogenic and/or probiotic bacteria at the serosal side level as shown in Table I.

Table I

	0h	2.5h	24h
BASAL (medium alone)			
EIEC	+EIEC		
EIEC + LCDG	+ EIEC	+ LCDG	
LCDG + EIEC	+LCDG	+ EIEC	
EIEC and LCDG	+EIEC and LCDG		

= CULTURE MEDIUM
 = EIEC
 = LCDG

The pathogenic bacteria used in the experiments were: Enteroinvasive *Escherichia coli* (EIEC); the probiotics tested were *Lactobacillus casei* DG[®] (*Lactobacillus paracasei* CNCM I1572; LCDG). The former was chosen for its harmful effects on the gastrointestinal tract, the latter for its probiotic effect.

The supernatants were subsequently recovered and the biopsies were treated with RIPA buffer. Both were frozen at -80°C.

Protein extraction was controlled by Western Blot as described in Turco, F.

et al. 2015. The antibodies used were: rabbit anti-iNOS (1:500 vol/vol) or mouse anti- α actin (1:1000 vol/vol).

Nitric oxide (NO) was measured using the same technique already described in Turco, F. et al. 2015.

In the case of the ELISA test (Enzyme-Linked ImmunoSorbent Assay) that was used to measure S100B, the protocol followed was the one described in the manufacturer's manual.

The statistical analyses included analysis of variance and the Bonferroni multiple comparison test. The data presented are the mean $\pm$ SD of the experiments. The level of statistical significance was fixed at $p < 0.05$.

Basal levels of iNOS expression and release of NO, S100B and Cytokines

The basal levels of expression and release of inflammation mediators in the gastrointestinal tract were measured.

Patients with diverticulosis showed a higher level of iNOS expression than healthy subjects (Fig. 1A).

Patients with symptomatic uncomplicated diverticular disease (SUDD) and symptomatic uncomplicated diverticular disease preceded by acute diverticulitis (SUDD+AD) showed greater differences in iNOS expression compared to healthy subjects (+2.04 and +2.86 times compared to the control -Fig. 1A).

Patients with asymptomatic diverticulosis and those with SUDD showed a slightly higher release of NO than healthy subjects (Fig. 1B). SUDD+AD patients showed a more marked difference (+7.77 times compared to the control - Fig. 1B).

The data shown in Fig. 1C indicate that there were no significant differences in the expression of the protein S100B (previously associated with the release of NO).

Furthermore, an increase in the release of the cytokine IL-1 β was demonstrated, above all in the SUDD+AD patients compared to healthy subjects (Fig. 2A). The same pattern was also observed for the cytokine IL-6 (Fig. 2B). As for IL-10, an increase in the level of release was

observed in particular in the SUDD+AD patients (Fig.2C).

Effects of bacterial stimulation on iNOS expression.

The effect on said values following bacterial stimulation on the expression of the inflammation mediator was subsequently tested both in healthy subjects and those with the various types of diverticulosis.

In particular, iNOS expression in mucosal biopsies was assessed.

In healthy (control) subjects, the stimulation with EIEC brought about an increase in iNOS expression (+2.11 compared to basal - Fig. 3A). The addition of LCDG before or after EIEC did not alter iNOS expression, whereas when LCDG was added together with EIEC, a significant reduction in iNOS expression was observed (-1.50 times compared to stimulation with EIEC alone - Fig. 3A).

In the patients with diverticulosis as well, the presence of EIEC increased iNOS expression (+2.86 times compared to basal – Fig. 3B). In this case the addition of LCDG brought about a reduction in the level of iNOS expression compared to stimulation with EIEC alone, irrespective of the moment in which the bacteria was added to EIEC (Fig. 3B).

In patients with SUDD and SUDD+AD, the stimulation with EIEC caused iNOS expression to increase (+2.14 compared to basal – Fig. 3C). In this case the action of LCDG was more effective if administered with EIEC (Fig.3C). Stimulation with LCDG did not in any case bring about an increase in the level of iNOS expression.

Effects of bacterial stimulation on the release of NO.

In the samples of healthy subjects, the release of NO increased significantly following stimulation with EIEC (Fig. 4A). The addition of LCDG before, after, or together with EIEC significantly altered the basal value of NO.

The same effect was observed in the patients with asymptomatic diverticulosis and SUDD. In fact, the administration of EIEC increased the release of NO and the presence of LCDG cancelled out this effect (Fig. 4B and 4C) irrespective of when LCDG was added.

The SUDD+AD patients showed a very high basal level of release of NO and thus the introduction of EIEC did not bring about a significant increase in these subjects. The addition of LCDG likewise did not bring about any change in the levels of release of NO due to the very high basal level (Fig. 4D).

In the controls, stimulation with EIEC provoked an increase in the release of S100B (about +2.7 times compared to basal, $p < 0.05$ – Fig. 5A). Simultaneous stimulation with LCDG and EIEC provoked a reduction in the increase of S100B following the administration of EIEC (-2.90 times compared to EIEC, $p < 0.05$ – Fig. 5A).

The results set forth above demonstrate that the activation of the inflammation system dependent on NO (and correlated with iNOS expression) leads to a release of NO which increases progressively going from subjects with diverticulosis to SUDD+AD. INOS expression follows the same trend. In particular, in diverticulosis (increase of 1.5 versus the control), in SUDD (increase of 2 versus the control) and in SUDD+AD (increase of 3 versus the control). Furthermore, it was also demonstrated that the administration of LCDG prevents the observed effects. In particular, LCDG is capable of countering the effects of pathogenic bacteria, particularly in subjects with SUDD+AD.

These data clearly demonstrate that LCDG can be effectively used as a probiotic for the prevention and/or treatment, in subjects with diverticulosis, in particular diverticular disease SUDD and SUDD+AD, and/or symptoms associated therewith.