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(54) **Title**: USE OF A COMPOSITION COMPRISING MICROORGANISMS TO INCREASE THE INTESTINAL PRODUCTION OF BUTYRIC ACID, FOLIC ACID OR NIACIN AND/OR DECREASE THE INTESTINAL PRODUCTION OF SUCCINIC ACID

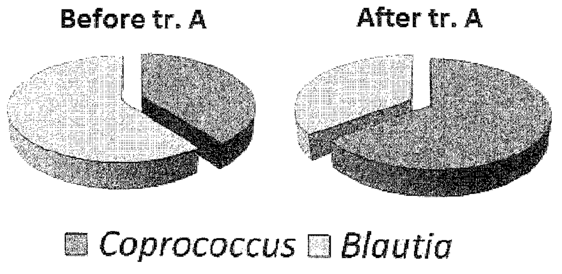

Fig.2.1

(57) **Abstract**: The present invention relates to the use of a composition comprising bacteria to increase the intestinal production of butyric acid, folic acid or niacin and/or to decrease the intestinal production of succinic acid. Moreover, the present invention relates to the use of said composition for the treatment and/or prevention of an intestinal butyrate- and/or succinate- dependent pathological condition. In particular, for the treatment and/or the prevention of intestinal inflammation, diarrhoea, ulcerative colitis or intestinal colopathies.

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DESCRIPTION

"USE OF A COMPOSITION COMPRISING MICROORGANISMS TO INCREASE THE INTESTINAL PRODUCTION OF BUTYRIC ACID, FOLIC ACID OR NIACIN AND/OR DECREASE THE INTESTINAL PRODUCTION OF SUCCINIC ACID"

The present invention relates to the use of a composition comprising bacteria in order to increase the intestinal production of butyric acid, folic acid or niacin and/or to decrease the intestinal production of succinic acid.

Moreover, the present invention relates to the use of said composition for the treatment and/or prevention of an intestinal butyrate- and/or succinate-dependent pathological condition, in particular, for the treatment and/or prevention of intestinal inflammation, diarrhoea, ulcerative colitis or intestinal colopathies.

Intestinal microbiota, also known by the by now obsolete term of intestinal flora, is the whole of the microorganisms, prevalently consisting of bacteria, residing in the intestine and in symbiosis with the body of the host.

The intestinal microbiota is a highly complex ecosystem and the condition of equilibrium among the different microorganisms making up the intestinal is fundamental in order to ensure the body's well-being and health, since the microbiota significantly conditions the development and the homeostasis of the intestinal mucosa of the host individual.

In other words, the intestinal microbiota represents a veritable organ. In fact, qualitative and/or quantitative modifications in the intestinal microbiota of an individual, or so-called disbiosis or dismicrobism, can result in the loss of the intestinal homeostasis, which in turn can condition the etiopathogenesis of a large number of pathologies.

For the purpose of treating a condition of intestinal disbiosis, or, in any case, for the purpose of maintaining the homeostasis of the intestinal microbiota, people often take substances that are defined as probiotics, or, according to the definition of the FAO/WHO, "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Similarly, the effectiveness of paraprobiotics for health has also been demonstrated; these are defined as "non-viable microbial cells (intact or broken) or raw cellular extracts which, when administered in adequate amounts (orally or topically), confer a health benefit on the host" (Taverniti and Guglielmetti, 2011).

It is clear that the beneficial activities of a microorganism will vary depending on the composition thereof and, in fact, these are often strain-specific activities.

On the basis of the above considerations, there continues to be a felt need to determine potential new health-promoting and/or therapeutic effects of microorganisms, in particular those included in a probiotic or in a paraprobiotic, in order to broaden the applications of use.

For example, there continues to be a greatly felt need in the art to identify microorganisms capable of modulating the intestinal amount of substances that are particularly beneficial and therapeutic for the body, such as butyric acid, folic acid and nicotinic acid.

Butyric acid is a short-chain fatty acid which is physiologically formed in the colon of humans as a result of the fermentation of dietary fibre by the microbiota.

Butyric acid is the principal source of energy for colon cells (colonocytes) and is therefore a nutrient that is essential for the human body.

At the intestinal level, butyric acid performs various important functions, e.g.: it stimulates the turnover and physiological maturation of colonocytes; it plays a key role in maintaining the integrity of the mucosa and in processes of repairing intestinal lesions; it stimulates the reabsorption of water and sodium in the colon; and it contributes to lowering the intestinal pH, creating an environment that is unfavourable to the development of pathogenic bacteria.

A deficiency of butyric acid can cause inflammatory colitis in humans.

Succinic acid is likewise a short-chain organic acid, of the bicarboxylic type. It is considered ulcerogenic and can cause serious damage to the mucosa. Therefore, an increase in the amount of succinic acid (succinate) is harmful to human health.

Folic acid (vitamin B9, or M or folacin) is a very important vitamin for the whole population, in particular in adults over 50 years of age and in women of a fertile age, because it intervenes (directly or, most of the time, by decreasing the plasma levels of homocysteine) in many vital processes such as DNA synthesis, repair and methylation.

A deficiency of folic acid can lead to macrocytic anaemia, which may be accompanied by leukopaenia and thrombocytopaenia, skin and mucosa alterations and gastrointestinal disorders (malabsorption and diarrhoea).

Niacin (or vitamin PP or vitamin B3), i.e. nicotinic acid and nicotinamide, is important because, among other things, it is the essential component of the coenzymes NAD and NADH and a deficiency thereof causes a pathology known as pellagra. Generally, this pathology begins with problems in the gastrointestinal system, which are then compounded by a photosensitizing dermatitis, mental disorders with fatigue, depression and memory disorders.

The present invention responds to the needs of the prior art described above with a composition comprising microorganisms, preferably bacteria of the genus *Lactobacillus* species *paracasei,* capable of (directly and/or indirectly) increasing, in an individual that takes it, the intestinal production of butyric acid, folic acid, niacin and/or salts thereof.

Furthermore, the Applicant has found, wholly unexpectedly, that a composition comprising microorganisms, preferably of the genus *Lactobacillus* species *paracasei*, is capable of (directly and/or indirectly) decreasing the intestinal production of succinic acid and/or salts thereof.

Therefore, the composition of the present invention is particularly advantageous for the treatment and/or prevention of intestinal butyrate- and/or succinate-dependent pathological conditions.

Further advantages of the present invention will be more apparent from the detailed description that follows and from the examples which, however, have only a demonstrative, non-limiting purpose.

To enable a better understanding of the detailed description, Figures 1-4 have been appended hereto:

- Figure 1.1 shows the result of the statistical analysis demonstrating the increase in the population of bacteria of the genus *Coprococcus* before and after treatment with the composition of the present invention (A) and the decrease thereof, in contrast, before and after treatment with the placebo (B);
- Figure 1.2 shows the result of the statistical analysis demonstrating the decrease in the population of bacteria of the genus *Blautia* before and after treatment with the composition of the present invention (A) and the increase thereof, in contrast, before and after treatment with the placebo (B);
- Figure 2.1 shows the increase in the population of bacteria of the genus *Coprococcus* (dark grey) and the decrease in the population of bacteria of the genus *Blautia* (light grey) before and after treatment with the composition of the present invention;
- Figure 2.2 shows the percentage increase in the population of bacteria of the genus *Coprococcus* (dark grey) and the percentage decrease in the population of bacteria of the genus *Blautia* (light grey) before and after treatment with the composition of the present invention (A) and the percentage decrease in the population of bacteria of the genus *Coprococcus* (dark grey) and the percentage increase in the population of bacteria of the genus *Blautia* (light grey) before and after treatment with the placebo (B);
- Figure 3 shows the result of the statistical analysis which demonstrates the increase in the metabolism of nicotinic acid before and after treatment with the composition of the present invention and the decrease thereof before and after treatment with the placebo;

- Figure 4 shows the result of the statistical analysis which demonstrates the increase in the biosynthesis of folic acid before and after treatment with the composition of the present invention and an absence of any modifications, in contrast, before and after treatment with the placebo.

The present invention relates to the use of a composition comprising microorganisms, preferably at least one bacterium of the genus *Lactobacillus* species *paracasei,* to increase the direct and/or indirect intestinal production of butyric acid and/or salts thereof, and/or folic acid and/or salts thereof, and/or niacin and/or salts thereof and/or to decrease the direct and/or indirect intestinal production of succinic acid and/or salts thereof.

In the context of the present invention, intestinal production means the release, into the environment, of any molecule produced by primary or secondary metabolism by any intestinal microorganism in any region of the intestine.

Moreover, the composition of the present invention can also be used to reduce the intestinal proliferation of pathogenic microorganisms, and/or to promote the integrity of the intestinal mucosa, and/or to promote the processes of repair of intestinal lesions, preferably by increasing the direct and/or indirect intestinal production of butyric acid and/or salts thereof and/or by decreasing the direct and/or indirect intestinal production of succinic acid and/or salts thereof.

Some pathogenic microorganisms particularly sensitive to the composition of the present invention are, for example, enterohaemorrhagic *Escherichia coli*, *Listeria monocytogenes*, *Clostridium difficile*, *Pseudomonas aeruginosa* and *Salmonella* spp.

The above-described uses of the composition of the present invention are intended both for a healthy individual and an individual with a pathological intestinal condition. In particular, in the case of a healthy individual, the composition of the invention performs in that individual, following intake, an action of maintaining the homeostasis of the microbiota and/or of preventing an alteration thereof, and is thus also definable as a probiotic composition (or probiotic).

A further aspect of the present invention relates to the medical use of the composition comprising microorganisms, preferably at least one bacterium of the genus *Lactobacillus* species *paracasei,* for the treatment and/or prevention of an intestinal butyrate- and/or succinate-dependent pathological condition.

In the context of the present invention, intestinal butyrate- and/or succinate-dependent pathological condition means a pathological condition that is sensitive to treatment with butyric acid and/or salts thereof and/or treatment with succinic acid and/or salts thereof. Examples of said pathologies are: diarrhoea, intestinal inflammation, ulcerative colitis, gastric atrophy, intestinal diverticula, stenosis, obstructions and diabetic neuropathy.

In a particularly preferred embodiment of the present invention, the composition comprises the bacterial strain *Lactobacillus paracasei* DG.

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

In a further embodiment of the invention, the direct and/or indirect increase in the intestinal production of butyric acid and/or salts thereof, and/or of folic acid and/or salts thereof, and/or of niacin and/or salts thereof and/or the direct and/or indirect decrease in the intestinal production of succinic acid is ascribable to the intestinal microbiota, preferably bacteria of the genus *Coprococcus* and/or *Blautia.*

In the particularly preferred embodiment of the invention, the direct and/or indirect increase in the intestinal production of butyric acid and/or salts thereof is ascribable to bacteria of the genus *Coprococcus*, and/or the direct and/or indirect decrease in the intestinal production of succinic acid is ascribable to bacteria of the genus *Blautia*.

Therefore, the composition comprising microorganisms, preferably at least one bacterium of the genus *Lactobacillus* species *paracasei*, more preferably the bacterial strain *Lactobacillus paracasei* DG, can also be used to modify the density of the bacterial population of the genus *Coprococcus* and/or *Blautia* in the intestinal microbiota, preferably so as to induce an increase in the bacterial population of the genus *Coprococcus* and/or a decrease in the bacterial population of the genus *Blautia*. In other words, intake of the composition of the present invention modifies the amount of bacteria of the genus *Coprococcus* and/or *Blautia* within the intestinal microbiota. In particular, the bacteria of the genus *Coprococcus* increase and/or the bacteria of the genus *Blautia* decrease following intake of said composition.

The composition used in the present invention comprises said microorganism, preferably said at least one bacterium of the genus *Lactobacillus* species *paracasei,* in live or dead form, as a lysate or extract.

In one embodiment of the invention, the composition comprises about 15-30 billion colony forming units (CFU) of bacteria, preferably 20-25 billion CFU of bacteria.

Preferably, the composition is formulated for oral administration. In particular, the composition is formulated in solid form, preferably in the form of pills, capsules, tablets, granular powder, hard capsules, water-soluble granules, sachets or pellets.

Alternatively, the composition of the invention is formulated in liquid form, for example as a syrup or beverage, or is added to a food, for example to a yogurt, cheese or fruit juice.

Alternatively, the composition of the invention is formulated in a form capable of exerting an action topically, for example as an enema.

In one embodiment of the invention, the composition further comprises excipients generally accepted for the production of probiotic and/or pharmaceutical products.

In a further embodiment of the invention, the composition of the invention can be enriched with vitamins, trace elements such as zinc and selenium, enzymes, prebiotic substances such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, guar gum or combinations thereof.

Preferably, for the purposes of the uses of the present invention, the composition is taken once a day, more preferably upon awakening. Alternatively, it can also be taken in the evening, preferably after meals.

EXAMPLE

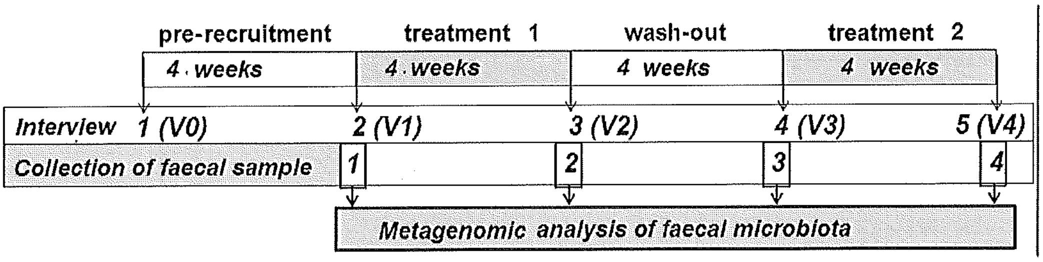
Treatment.

A randomized, double-blind, placebo-controlled crossover dietary intervention study was conducted on healthy individuals.

Volunteers were recruited in accordance with the following criteria:

- inclusion criteria: healthy men and women, ranging in age between 18 and 55 years who gave their informed consent;
- exclusion criteria: antibiotic treatment in the month preceding the first examination; episodes of viral or bacterial enteritis in the 2 months preceding the first examination; gastric or duodenal ulcers in the 5 years preceding the first examination; pregnancy or breastfeeding; recent or presumed cases of alcoholism and drug intake; other conditions of non-compliance with the study protocol.
- The probiotic dietary intervention was carried out in accordance with a design crossover, as schematized in Table I below.

Table I



In the pre-enrolment phase (4 weeks) the volunteers followed their usual diet, without consuming probiotic fermented milk products (traditional yogurt was thus permitted), probiotic dietary supplements, or prebiotic dietary supplements.

At the end of the pre-enrolment period, the volunteers were randomized to receive one capsule per day of a probiotic or placebo for 4 weeks.

By way of example, Enterolactis Plus was used as the probiotic to be administered; it consists in 420 mg capsules containing 24 billion CFU (colony forming units) of *Lactobacillus paracasei* strain DG.

The placebo consisted in capsules identical in appearance to the probiotic ones, obviously devoid of the probiotic agent.

The flavour and colour of the active substance (i.e. the probiotic) and the placebo were identical.

The product was taken in the morning on an empty stomach, at least ten minutes before breakfast or, if forgotten, in the evening before going to bed and in any case at least two hours after the last meal.

After the first four weeks of treatment, the volunteers went through a four-week wash-out period identical to the pre-enrolment period.

At the end of the wash-out period, the volunteers took one capsule per day of Enterolactis Plus or placebo for four weeks in accordance with the crossover design described above.

In summary, the study involved 4 phases, each of which lasting 4 weeks:

- *Pre-recruitment phase*: the individuals underwent neither treatment A nor treatment B.
- *Treatment 1*: the individuals underwent treatment A or treatment B.
- *Wash-out*: the individuals underwent neither treatment A nor treatment B
- *Treatment 2*: the individuals underwent treatment B or treatment A.

Treatments A and B can be the composition of the present invention, in the specific example Enterolactis plus, or else the placebo. At the start of the treatment, it was not known what the individual was taking; only at the end of the treatment, when the blind was broken, was the intake sequence known.

Examinations and sample collection.

Each volunteer was initially instructed as to the entire procedure to be followed, which involved a total of 5 meetings per volunteer.

During the first meeting, informed consent was obtained along with the volunteer's personal data. The volunteer also received general information about how the study was to be carried out and was instructed about the changes in the diet to be applied in the subsequent 4 weeks of pre-enrolment (prohibition from consuming the previously specified products).

After 4 weeks, the volunteer went to the second meeting with a faecal sample (sample T0), collected during the previous 24 hours in a special container handed over during the first meeting.

To ensure optimal preservation, the faecal samples were stored at room temperature and delivered to the laboratory within 24 hours.

During the second meeting, moreover, the volunteer was given the probiotic product (or placebo) to be taken during the next 4 weeks. Moreover, the volunteer was instructed as to how to take the product.

At the end of the 4 weeks of taking the product (or placebo), the volunteer went to the third meeting with another faecal sample (sample T1) collected during the previous 24 hours.

During the third meeting, the volunteer completed a questionnaire on the possible effects, both positive and undesirable ones, deriving from consumption of the product.

The volunteer was then instructed about the next 4 weeks, during which he or she again did not take the previously mentioned products.

At the end of these 4 weeks, the volunteer went to the fourth meeting with a faecal sample (sample T2) and received the probiotic product (or placebo) to be taken during the next 4 weeks.

Finally, after 4 weeks of taking the product (or placebo), the volunteer went to the fifth meeting to deliver the last faecal sample (sample T3).

During this last meeting, the volunteer has completed a questionnaire analogous to the one received during the third meeting.

All the faecal samples collected were stored at -20°C for no more than 7 days before being subjected to analysis of the microbiota.

Analysis of faecal microbiota

The faecal microbiota was evaluated by analyzing the nucleotide sequence of portions of the gene encoding the 16S rRNA bacterial ribosomal subunit. More specifically, a metagenomic strategy was adopted; it consists in short in the following steps:

1. extracting, quantifying and normalizing the metagenomic DNA from the faecal samples;
2. amplifying the V3 hypervariable region of the bacterial gene encoding the 16S rRNA by PCR;
3. quantifying the PCR products;
4. sequencing the amplification products;
5. bioinformatically analyzing the sequences.

The procedures according to steps 1 and 3 are techniques that are well known in the art and they are thus performed with the protocols commonly used in this field. For example, the methods described in laboratory manuals such as those by Sambrook et al. 2001, or Ausubel et al. 1994.

Step 2 of amplifying the V3 region of the 16S ribosomal RNA genes was performed by means of the DNA amplification technique known as PCR, using Probio_Uni 5'-CCTACGGGRSGCAGCAG-3' (SEQ ID NO: 1) and Probio_Rev 5'-ATTACCGCGGCTGCT-3' (SEQ ID NO: 2) as oligonucleotides (primers).

In particular, the pair of primers SEQ ID NO: 1 and 2 2 amplifies the V3 region of the 16S rRNA gene.

Step 4 can be performed with the techniques known in the art for this purpose, for example techniques based on the Sanger method, pyrosequencing or the Ion Torrent Fusion Primers sequencing method used in the specific example of the present invention according to the protocol described in the materials and methods section of the scientific article by Milani et al. (2013).

In the case of the Ion Torrent technique, the primers are designed and synthesized in such a way as to include, at the 5' end, one of the two adaptor sequences used in this specific DNA sequencing technique. In this case, the adaptor sequences were SEQ ID NO: 1 and 2.

The conditions under which the PCR was performed are the following:

- 5 minutes at 95°C;
- 30 seconds at 94°C, 30 seconds at 55°C, and 90 seconds at 72°C for 35 cycles;
- 10 minutes at 72°C.

At the end of the PCR, the integrity of the amplificate was verified by electrophoresis.

Step 5 of the method, necessary for characterizing the microbial communities, can be carried out with numerous techniques presently known for this purpose. More specifically, use was made of: hierarchical clustering, taxonomic analysis and construction of phylogenetic dendrograms with heat maps according to the protocol described in the materials and methods section of the scientific article by Milani et al. (2013); more specifically, the analysis of sequence data was conducted using QIIME software.

Statistical analysis of the data

The statistical analysis was conducted using STATISTICA software (Statsoft Inc., Tulsa, OK, USA).

In order to reveal significant differences, the data were analyzed using both parametric (multivariate and univariate repeated-measures ANOVA) and non-parametric (Wald-Wolfowitz and Mann-Whitney) statistical methods.

The normality of the data series (important assumption for ANOVA) was evaluated by means of the Shapiro – Wilk and Kolmogorov-Smirnov tests.

Results of the treatment

The study was completed by a total of 22 individuals (11 females and 11 males).

Thirty-three individuals were initially enrolled, but 11 of them withdrew early for various reasons: intake of antibiotics (4), refusal to continue the study (1), frequent episodes of diarrhoea (1), intake of other probiotics during the study period (3), drastic change in eating habits (1), and seasonal influenza with episodes of diarrhoea (1).

Upon the conclusion of the study and completion of the analysis of the results of the two treatments, the blind was broken and it was seen that: treatment A is the active treatment, containing *Lactobacillus paracasei* DG; treatment B is the placebo, identical on the exterior to the active treatment, but devoid of lactobacilli.

When the data obtained from the study were analyzed, a high stability, from a taxonomic viewpoint, of the intestinal microbiota of the study participants was observed.

In fact, it was found that:

- Two bacterial divisions of the 15 identified, namely, Bacteroidetes and Firmicutes, constitute over 90% of the sequences;
- 11 families of the 131 identified constitute over 90% of the sequences; and
- 20 genera of the 262 identified constitute over 90% of the sequences.

Moreover, this study confirmed that human intestinal microbiota at lower taxonomic levels (i.e. at the family and genus levels) is highly variable from one individual to another.

Therefore, the experimental evidence demonstrated the necessity of conducting, on a healthy population, crossover intervention trials in order to prevent the marked inter-individual variability from hiding the possible effects of the probiotic treatment or leading to false statistical positives.

When the modifications induced in the intestinal microbiota by the two treatments were evaluated, a statistically significant difference emerged in terms of genera only in the group receiving the treatment with *Lactobacillus paracasei* DG (active treatment). More specifically, an increase in the genus *Coprococcus* was observed. In fact, as can be noted in Figures 1.1, 2.1 and 2.2, before and after treatment with *Lactobacillus paracasei* DG a statistically significant increase in coprococci was observed. In contrast, a moderate reduction thereof was seen in the group receiving the placebo treatment.

Moreover, after treatment with *Lactobacillus paracasei* DG, a statistically significant reduction in bacteria of the genus *Blautia* was observed. In contrast, a slight increase thereof was observed in the group receiving the placebo treatment (Figures 1.2, 2.1 and 2.2)

Coprococci are among the main producers of butyrate at the intestinal level.

Butyrate is a fundamental compound at the intestinal level, since on the one hand it contributes to restoring the functional integrity of the intestinal mucosa and maintaining it over time, and on the other hand it has important anti-inflammatory effects, so much so that it is used as an adjuvant to dietary treatments for intestinal colopathies (e.g. chronic inflammatory intestinal diseases).

Moreover, an analysis of their genome reveals that these bacteria can use succinate as a fermentation substrate.

This information is fundamental, in consideration of the fact that members of the genus *Blautia* generate acetate and succinate as main end products of the fermentation of glucose.

Succinate is considered an ulcerogenic factor, capable, therefore, of exacerbating the condition of individuals with ulcerative colitis, since it is probably to blame for the mucosal damage present above all in the active phases of the disease.

In conclusion, following treatment with a probiotic, in this case following the administration of *Lactobacillus paracasei* DG, one observes an increase in the bacteria belonging to the genus *Coprococcus* and hence an increase in the intestinal concentration of butyrate.

At the same time, one observes a reduction in the concentration of succinate, which may be to blame for mucosal damage in individuals with ulcerative colitis, in a direct manner, because following treatment with the probiotic, in this case following the administration of *Lactobacillus paracasei* DG, there is a reduction in the bacteria belonging to the genus *Blautia*, and, in an indirect manner, because the increased population of coprococci is further able to decrease the concentration of succinate by using it as a substrate in their fermentation process.

In conclusion, following treatment with the probiotic, in the specific example following the administration of *Lactobacillus paracasei* DG, there is an increase in the concentration of butyric acid in the faeces of individuals, with a simultaneous reduction in other organic acids, such as succinic acid.

The data relating to the composition of faecal microbiota were used, finally, in a bioinformatic analysis aimed at a virtual reconstruction of the metagenome based on knowledge of the bacterial genomes (Okuda S, Tsuchiya Y, Kiriyama C, Itoh M, Morisaki H. Virtual metagenome reconstruction from 16S rRNA gene sequences. Nat Commun. 2012;3:1203); in other words it was established in silico which potential genes are present and how abundantly in a given microbiota. This analysis made it possible to verify a putative increase in the encoding genes for the synthesis of folic acid and metabolism of nicotinic acid (Figures 3 and 4). These two molecules represent important vitamins for the human host (respectively named vitamin B9 and B3). Vitamin B9, in particular, represents a nutritional factor of primary importance, a deficiency of which, especially in specific physiological conditions such as pregnancy, can lead to serious health consequences. Treatment with the probiotic used in this study could therefore favor the ability of intestinal microbiota to produce folic acid (vitamin B9), with a consequent nutritional benefit for the human host.

CLAIMS

1. Use of a composition comprising at least one bacterium of the genus *Lactobacillus* species *paracasei* for increasing the direct and/or indirect intestinal production of butyric acid and/or salts thereof, and/or folic acid and/or salts thereof, and/or niacin and/or salts thereof and/or for decreasing the direct and/or indirect intestinal production of succinic acid and/or salts thereof.
2. Use according to claim 1 for reducing the intestinal proliferation of pathogenic microorganisms, and/or for promoting the integrity of the intestinal mucosa, and/or for promoting the processes of repair of intestinal lesions by said direct and/or indirect increase in the intestinal production of butyric acid and/or salts thereof and/or by said direct and/or indirect decrease in the intestinal production of succinic acid and/or salts thereof.
3. A composition comprising at least one bacterium of the genus *Lactobacillus* species *paracasei* for use in the treatment and/or prevention of an intestinal butyrate- and/or succinate-dependent pathological condition.
4. The composition according to claim 3, wherein said intestinal pathological condition is selected from: diarrhoea, intestinal inflammation, ulcerative colitis, gastric atrophy, intestinal diverticula, stenosis, obstructions and diabetic neuropathy.
5. Use according to claim 1 or 2, or the composition according to claim 3 or 4, wherein said Lactobacillus is the strain *Lactobacillus paracasei* DG.
6. Use according to any one of claims 1, 2 and 5 , wherein said direct and/or indirect increase in the intestinal production of butyric acid and/or salts thereof, and/or folic acid and/or salts thereof, and/or niacin and/or salts thereof and/or said direct and/or indirect decrease in the intestinal production of succinic acid is ascribable to

the intestinal microbiota, preferably it is ascribable to bacteria of the genus Coprococcus and/or Blautia.

7. Use according to any one of claims 1, 2, 5 and 6, wherein said direct and/or indirect increase in the intestinal production of butyric acid and/or salts thereof is ascribable to bacteria of the genus Coprococcus, and/or said direct and/or indirect decrease in the intestinal production of succinic acid is ascribable to bacteria of the genus Blautia .
8. Use according to any one of claims 1, 2, 5-7, or the composition according to claim 3 or 4, wherein said at least one bacterium of the genus *Lactobacillus* species *paracasei* is a live or a dead bacterium, or a bacterial lysate or extract.
9. Use according to any one of claims 1, 2, 5-8, or the composition according to any one of claims 3, 4 , 8, wherein said composition is for oral administration, preferably in the form of pills, capsules, tablets, granular powder, hard capsules, water-soluble granules, sachets or pellets.
10. Use according to any one of claims 1, 2, 5-9, or the composition according to any one of claims 3, 4, 8, 9, wherein said composition further comprises dietary fibres having prebiotic activity, preferably FOS, inulin or guar gum, or comprises other substances such as vitamins, trace elements and/or enzymes.

Fig.1.1

Coprococcus ssp.

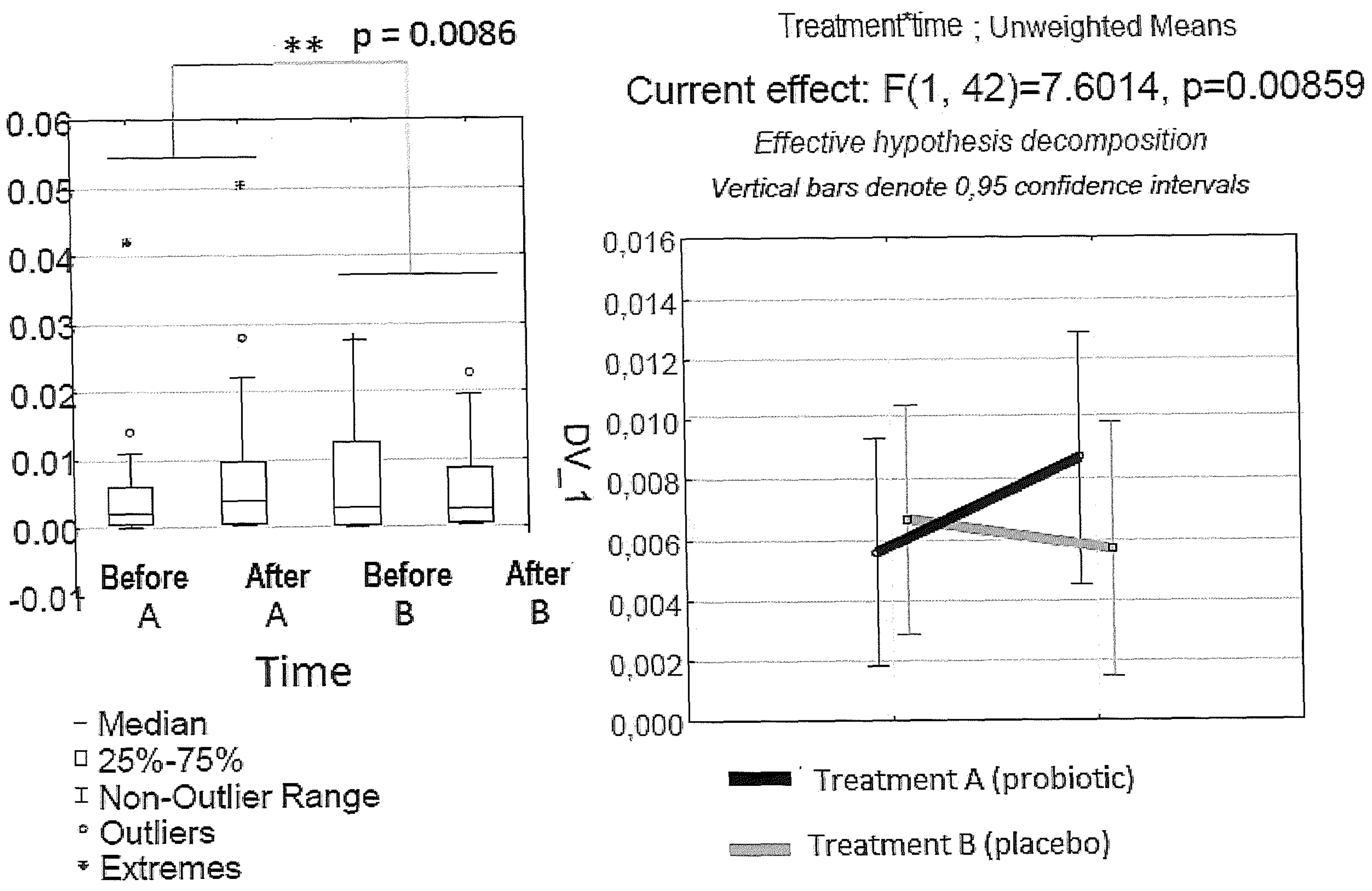
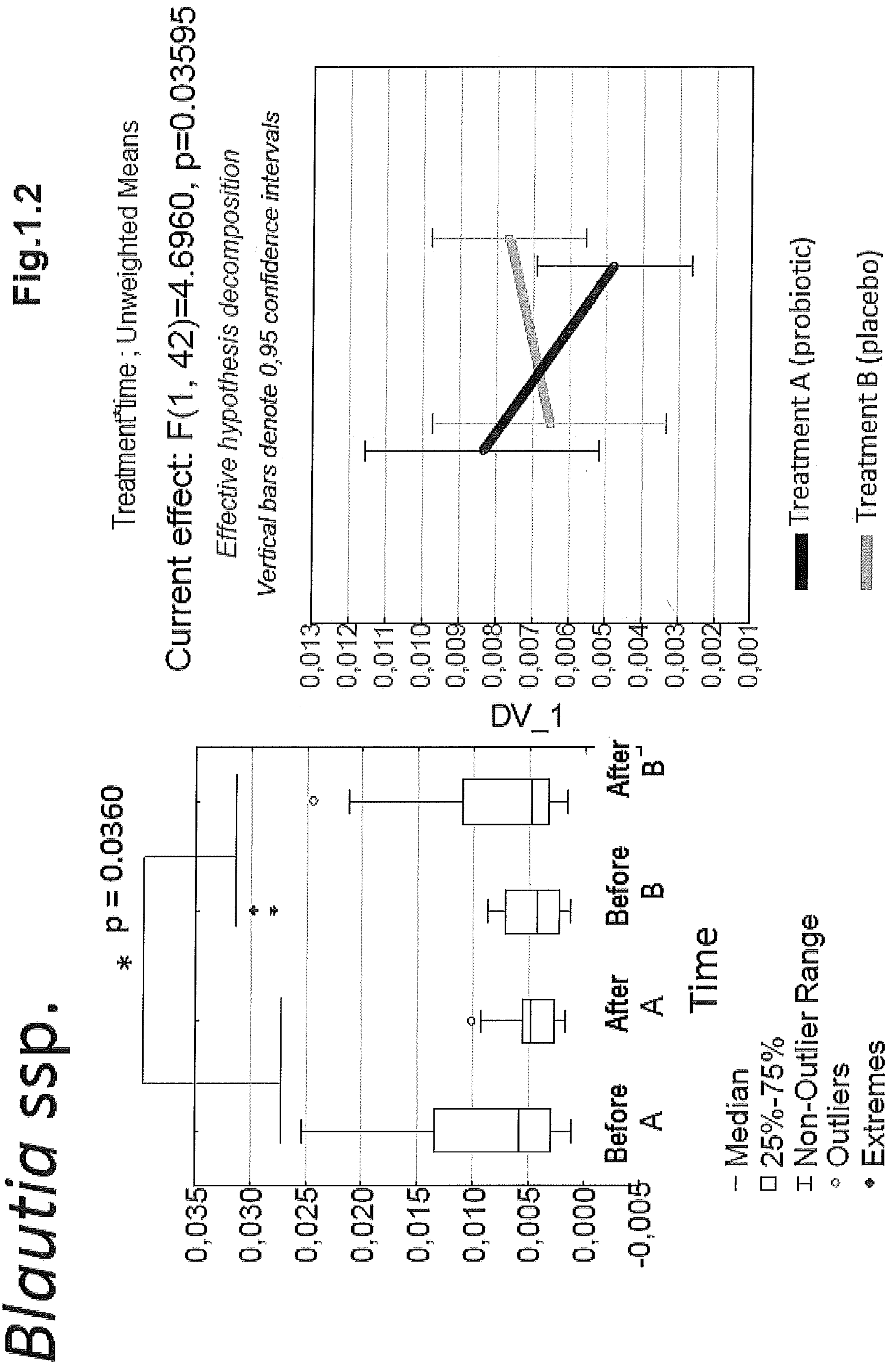


Fig.1.2


Blautia ssp.
* p = 0.0360
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
-0,005
Before A
After A
Before B
After B
Time
Median
25%-75%
Non-Outlier Range
Outliers
Extremes
Treatment*time ; Unweighted Means
Current effect: F(1, 42)=4.6960, p=0.03595
Effective hypothesis decomposition
Vertical bars denote 0,95 confidence intervals
DV_1
0,013
0,012
0,011
0,010
0,009
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
Treatment A (probiotic)
Treatment B (placebo)

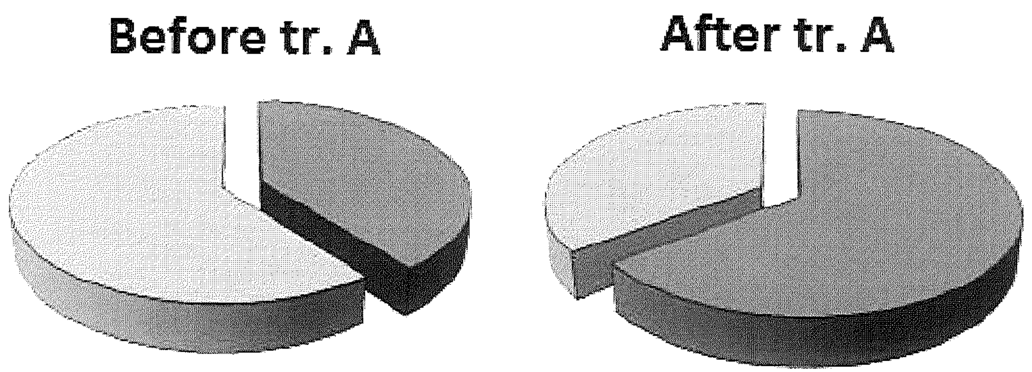

Before tr. A
After tr. A

Fig.2.1

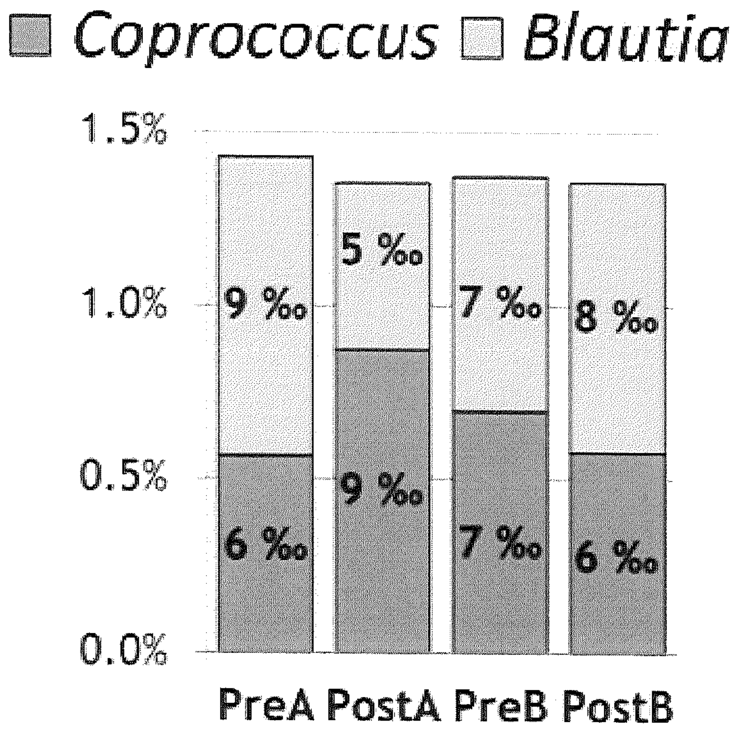

Coprococcus
Blautia
1.5%
1.0%
0.5%
0.0%
9 ‰
6 ‰
5 ‰
9 ‰
7 ‰
7 ‰
8 ‰
6 ‰
PreA
PostA
PreB
PostB

Fig.2.2

Fig.3

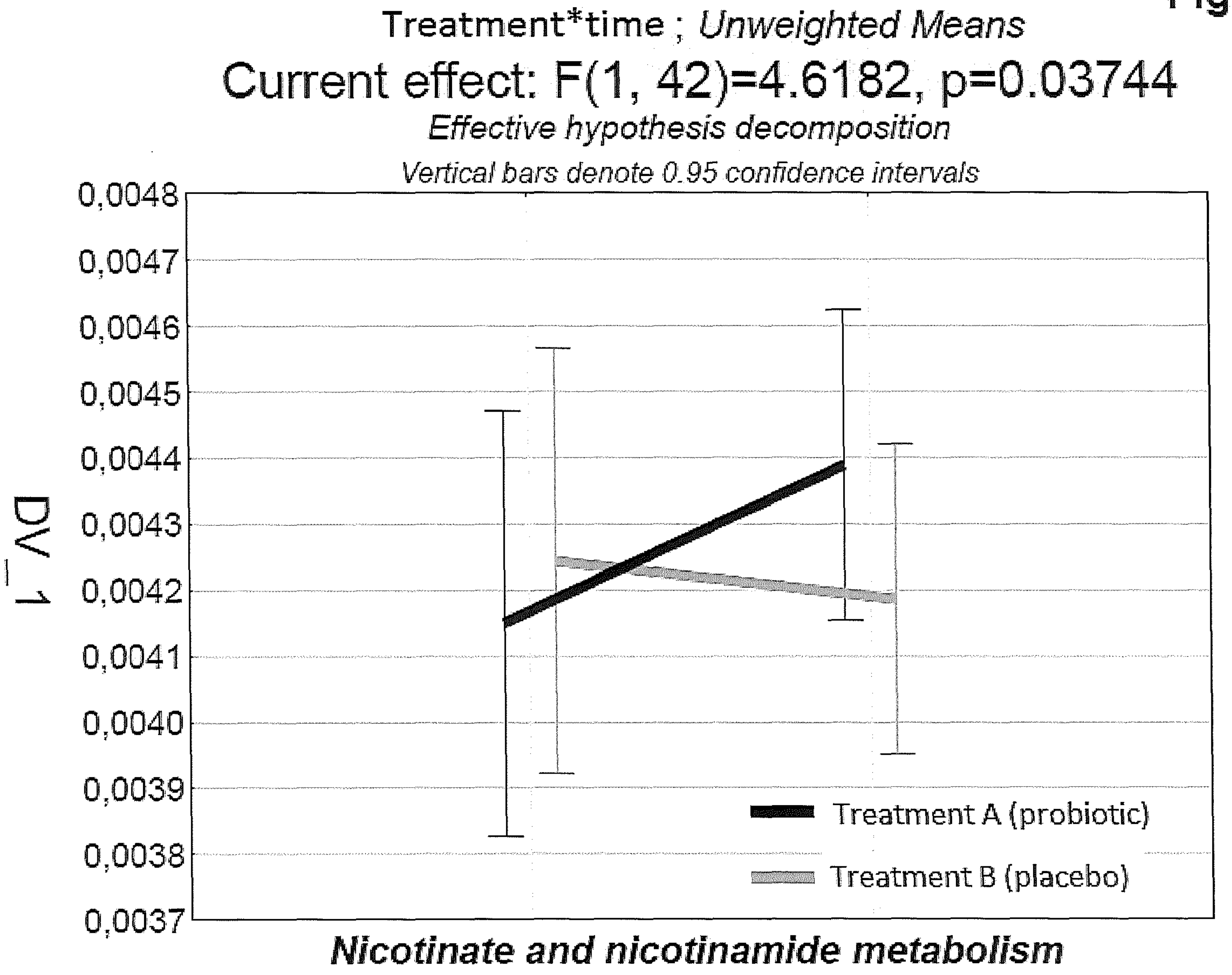
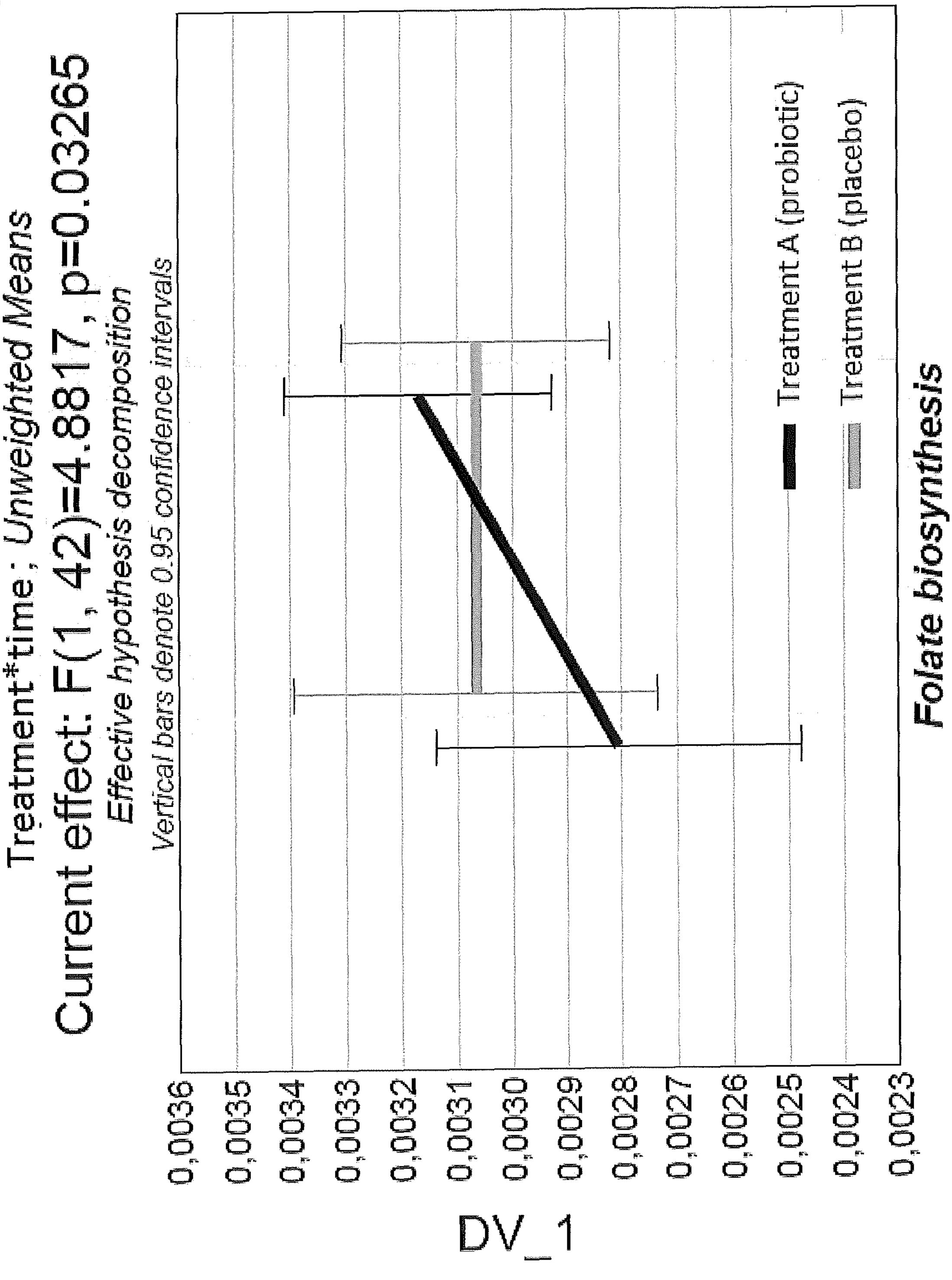

Treatment*time ; Unweighted Means
Current effect: F(1, 42)=4.6182, p=0.03744
Effective hypothesis decomposition
Vertical bars denote 0.95 confidence intervals
DV_1
0,0048
0,0047
0,0046
0,0045
0,0044
0,0043
0,0042
0,0041
0,0040
0,0039
0,0038
0,0037
Treatment A (probiotic)
Treatment B (placebo)
Nicotinate and nicotinamide metabolism

Fig.4


Treatment*time; Unweighted Means
Current effect: F(1, 42)=4.8817, p=0.03265
Effective hypothesis decomposition
Vertical bars denote 0.95 confidence intervals
DV_1
0,0036
0,0035
0,0034
0,0033
0,0032
0,0031
0,0030
0,0029
0,0028
0,0027
0,0026
0,0025
0,0024
0,0023
Treatment A (probiotic)
Treatment B (placebo)
Folate biosynthesis

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/064285

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/74 A61P1/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANUEL OLIVEIRA ET AL: "Lactobacillus paracasei Reduces Intestinal Inflammation in Adoptive Transfer Mouse Model of Experimental Colitis", CLINICAL AND DEVELOPMENTAL IMMUNOLOGY, vol. 23, no. 5, 1 January 2011 (2011-01-01), pages 1077-13, XP055086843, ISSN: 1740-2522, DOI: 10.1128/IAI.69.4.2277-2285.2001	1-4,6-10
Y	page 10, column 2, paragraph 2 - page 11, column 2, paragraph 1 ----- -/--	5

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 21 January 2015	Date of mailing of the international search report 29/01/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Zellner, Eveline

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/064285

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOMBARDO L: "New insights into Lactobacillus and functional intestinal disorders", MINERVA GASTROENTEROLOGICA E DIETOLOGICA, EDIZIONI MINERVA MEDICA, TORINO, IT, vol. 54, no. 3, 1 September 2008 (2008-09-01), pages 287-293, XP009117218, ISSN: 1121-421X	1-4,6-10
Y	page 290, column 1, paragraph 2 - column 2, paragraph 3 -----	5
X	WO 00/54788 A1 (ITALMED S N C DI GALLI G E PAC [IT]; GALLI GIOVANNA [IT]) 21 September 2000 (2000-09-21)	1-4,6-10
Y	page 8, line 27 - page 9, line 6; claims 1,5,7,8 -----	5
X	FRANCESCA VALERIO: "Effects of Probiotic Lactobacillus paracasei-enriched Artichokes on Constipated Pateients", J CLIN GASTROENTEROL, 10 September 2010 (2010-09-10), pages 49-43, XP009174008,	1-4,6-10
Y	abstract -----	5
E	WO 2015/000972 A1 (SG AUSTRIA PTE LTD [SG]; GUENZBURG WALTER H [SG]) 8 January 2015 (2015-01-08) paragraphs [0051], [0065]; claims 17,33 -----	1-10
E	WO 2014/137211 A1 (UNIV GRONINGEN [NL]; GRONINGEN ACAD ZIEKENHUIS [NL]; UNIV WAGENINGEN [] 12 September 2014 (2014-09-12) page 8, line 6 - page 9, line 33 -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2014/064285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0054788 A1	21-09-2000	AT 228001 T	15-12-2002
		AU 3555800 A	04-10-2000
		DE 60000825 D1	02-01-2003
		DE 60000825 T2	04-09-2003
		EP 1162987 A1	19-12-2001
		ES 2188521 T3	01-07-2003
		IT FI990051 A1	15-09-2000
		WO 0054788 A1	21-09-2000
WO 2015000972 A1	08-01-2015	NONE	
WO 2014137211 A1	12-09-2014	NONE	

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(83)生物保藏信息
CNCM I-1572 1995.05.05

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(74)专利代理机构 上海专利商标事务所有限公司 31100
代理人 陶启长 杨昀
(51)Int.Cl.
A61K 35/747(2015.01)
A61P 1/00(2006.01)

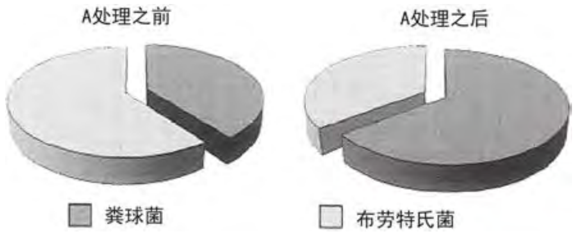
权利要求书1页 说明书7页 附图5页

(54)发明名称

含微生物的组合物在增加丁酸、叶酸或烟酸的肠道生产和/或降低琥珀酸的肠道生产中的应用

(57)摘要

本发明涉及使用含细菌的组合物来提高丁酸、叶酸或烟酸的肠道生产和/或降低琥珀酸的肠道生产。此外，本发明涉及使用所述组合物治疗和/或预防肠道丁酸盐依赖性和/或琥珀酸盐依赖性的病理状况。具体用于治疗和/或预防肠道炎症、腹泻、溃疡性结肠炎或肠道结肠病。



权 利 要 求 书

1.含乳杆菌(Lactobacillus)属副干酪(paracasei)种的至少一种细菌的组合物在增加丁酸和/或其盐、和/或叶酸和/或其盐、和/或烟酸和/或其盐的直接和/或间接肠道生产和/或降低琥珀酸和/或其盐的直接和/或间接肠道生产中的用途。

2.如权利要求1所述的用途,所述用途用于通过丁酸和/或其盐的所述直接和/或间接肠道生产的增加和/或通过琥珀酸和/或其盐的所述直接和/或间接肠道生产的降低,减少致病微生物的肠道增殖、和/或用于提升肠道黏膜的完整性、和/或促进肠道损伤的修复过程。

3.一种组合物,所述组合物包含乳杆菌属副干酪种的至少一种细菌,用于治疗和/或预防肠道丁酸盐-和/或琥珀酸盐-依赖性病理状况。

4.如权利要求3所述的组合物,其中所述肠道病理状况选自:腹泻、肠道炎症、溃疡性结肠炎、胃萎缩、肠憩室、狭窄、阻塞和糖尿病神经病变。

5.如权利要求1或2所述的用途或权利要求3或4所述的组合物,其中所述乳杆菌是副干酪乳杆菌DG株。

6.如权利要求1、2和5中任一项所述的用途,其中丁酸和/或其盐、和/或叶酸和/或其盐、和/或烟酸和/或其盐的所述直接和/或间接肠道生产的增加和/或琥珀酸的所述直接和/或间接肠道生产的降低归因于肠道微生物群,优选归因于粪球菌属和/或布劳特氏菌属的细菌。

7.如权利要求1、2、5和6中任一项所述的用途,其中丁酸和/或其盐的所述直接和/或间接肠道生产的增加归因于粪球菌属的细菌,和/或琥珀酸的所述直接和/或间接肠道生产的降低归因于布劳特氏菌属的细菌。

8.如权利要求1、2、5-7中任一项所述的用途或权利要求3或4所述的组合物,其中所述乳杆菌属副干酪种的至少一种细菌为活细菌或死细菌、或细菌裂解物或提取物。

9.如权利要求1、2、5-8中任一项所述的用途或权利要求3、4、8中任一项所述的组合物,其中所述组合物用于口服给予,优选为下述形式:丸剂、胶囊剂、片剂、粒状粉末、硬胶囊、水溶性颗粒、囊剂或粒料。

10.如权利要求1、2、5-9中任一项所述的用途或权利要求3、4、8、9中任一项所述的组合物,其中所述组合物还含有益生元活性的膳食纤维,优选FOS、菊粉、瓜尔豆胶,或者含有其他物质如维生素、微量元素和/或酶。

含微生物的组合物在增加丁酸、叶酸或烟酸的肠道生产和/或降低琥珀酸的肠道生产中的应用

[0001] 本发明涉及使用含细菌的组合物从而提高丁酸、叶酸或烟酸的肠道生产和/或降低琥珀酸的肠道生产。此外，本发明涉及使用所述组合物治疗和/或预防肠道丁酸盐依赖性和/或琥珀酸盐依赖性的病理状况，具体用于治疗和/或预防肠道炎症、腹泻、溃疡性结肠炎或肠道结肠病。

[0002] 肠道微生物群，旧称肠道菌丛，是肠道中居住的与宿主身体共生的微生物总和，主要由细菌组成。

[0003] 肠道微生物群是一个高度复杂的生态系统，组成肠道的不同微生物之间的平衡是确保身体健康的基础，因为微生物群会显著地调节宿主个体的肠道黏膜的发育和稳态。

[0004] 换言之，所述肠道微生物群相当于一个真实的器官。事实上，个体的肠道微生物群的定性和/或定量变化，或所谓失调或微生物失调(dismicrobism)，会导致肠道内稳态丧失，由此会决定大量病理学的发病机理。

[0005] 为了治疗肠道腹泻的症状或在任何情况写维持肠道微生物群的稳态，常摄取称为益生菌的物质，或者，按照FAO/WHO的定义，益生菌是一组″活的微生物，当以适当的量给予这些微生物时，能为宿主提供健康益处″。类似地，副益生菌(paraprobiotics)对健康的有效性也已得到证明；其被称为“非活力微生物细胞(完整或破损)或原始细胞提取物，将其以足够量(口服或局部)给予时对宿主健康产生益处”(Taverniti和Guglielmetti,2011)。

[0006] 很明显微生物的有益活性会基于其组合物而变化，事实上常存在株系特异性活性。

[0007] 基于上述考虑，仍需要确定微生物的可能的新型促进健康和/或治疗的效果从而开拓应用，尤其是益生菌或副益生菌中所具有的那些。

[0008] 例如，本领域仍特别需要鉴定能调控对身体特别有益和有治疗性的肠道物质(如丁酸、叶酸和烟酸)含量的微生物。

[0009] 丁酸是短链脂肪酸，其于生理学上在人类的结肠中通过微生物群对膳食纤维的发酵而形成。

[0010] 丁酸是结肠细胞(colonocytes)的主要能量来源，并且因此是对人体非常重要的营养物质。

[0011] 在肠道水平，丁酸行使各种重要的功能，例如：其刺激结肠细胞的更新和生理成熟；其在维持黏膜完整性和修复肠道损伤过程中起关键作用；其刺激结肠中水和钠的再吸收；并且其对降低肠道pH、形成对致病菌的发展不利的环境均有贡献。

[0012] 缺乏丁酸可导致人类的结肠炎。

[0013] 琥珀酸同样是二羧基型的短链有机酸。考虑其具有溃疡性并且可会黏膜造成严重损伤。因此，琥珀酸(琥珀酸盐)含量的增加对人体健康有害。

[0014] 叶酸(维生素B9、或M或叶酸(folacin))是对全部群体来说都很重要的维生素，尤其是50岁以上的成人和育龄女性，这是因为其介入(直接介入或在大多数情况中通过降低高半胱氨酸的血浆水平介入)许多至关重要的过程例如DNA合成、修复和甲基化。

[0015] 缺乏叶酸可导致巨红细胞性贫血，其可伴随有白斑病和血小板减少症、皮肤和黏膜变质和胃肠道紊乱（吸收不良和腹泻）。烟酸（或维生素PP或维生素B3）即尼克酸或烟碱，其非常重要，因其是辅酶NAD和NADH的必要组分之一，它们的缺乏会导致糙皮病病理学。通常，该病理学起始于胃肠道系统的问题，然后其伴随有光敏性皮炎、具疲劳的精神紊乱、抑郁和记忆紊乱。

[0016] 本发明响应上述的本领域需求，涉及包含微生物的组合物，所述微生物优选乳杆菌（Lactobacillus）属副干酪（paracasei）种的细菌，其能够（直接和/或间接）增加对其进行摄取的个体中丁酸、叶酸、烟酸和/或其盐的肠道生产。

[0017] 此外，发明人意外发现包含微生物（优选乳杆菌属副干酪种的细菌）的组合物能够（直接和/或间接）降低琥珀酸和/或其盐的肠道生产。因此，本发明组合物对治疗和/或预防肠道丁酸盐依赖性和/或琥珀酸盐依赖性的病理状况尤其有效。

[0018] 本发明的其它益处将根据下文的详细描述和实施例而更为显见，然而，其仅仅起说明而非限制作用。

[0019] 为了能够更好地理解详细说明，本发明附有图1-4：

[0020] -图1.1显示统计学分析的结果，证明用本发明组合物处理之前和之后粪球菌（Coprococcus）属细菌群体的增加（A），和用安慰剂处理之前和之后粪球菌属细菌群体的减少（B）；

[0021] -图1.2显示统计学分析的结果，证明用本发明组合物处理之前和之后布劳特氏菌（Blautia）属细菌群体的减少（A），和用安慰剂处理之前和之后粪球菌属细菌群体的增加（B）；

[0022] -图2.1显示，在用本发明组合物处理之前和之后，粪球菌属的细菌群的增加（深灰）和布劳特氏菌属的细菌群的减少（浅灰）；

[0023] -图2.2显示，用本发明组合物处理之前和之后（A），粪球菌属的细菌群的百分比增加（深灰）和布劳特氏菌属的细菌群的百分比减少（浅灰），以及用安慰剂处理之前和之后（B），粪球菌属的细菌群的百分比减少（深灰）和布劳特氏菌属的细菌群的百分比增加（浅灰）；

[0024] -图3显示统计学分析的结果，用于证明用本发明组合物处理之前和之后烟酸代谢的增加，和用安慰剂处理之前和之后烟酸代谢的减少；

[0025] -图4显示统计学分析的结果，用于证明用本发明组合物处理之前和之后，叶酸的生物合成的增加，和相反地用安慰剂处理之前和之后没有任何变化。

[0026] 本发明涉及含微生物（优选至少一种乳杆菌属副干酪种的细菌）的组合物在增加丁酸和/或其盐、和/或叶酸和/或其盐、和/或烟酸和/或其盐的直接和/或间接肠道生产，和/或降低琥珀酸和/或其盐的直接和/或间接肠道生产中的用途。

[0027] 本文中，肠道生产表示任何肠道微生物在肠道的任何区域通过初级或次级代谢产生的任何分子释放到环境中。

[0028] 此外，本发明组合物还可用于减少致病微生物的肠道增殖、和/或用于提升肠道黏膜的完整性、和/或促进肠道损伤的修复过程，优选通过增加丁酸和/或其盐的直接和/或间接肠道生产和/或通过降低琥珀酸和/或其盐的直接和/或间接肠道生产发挥上述作用。

[0029] 对本发明组合物特别敏感的一些致病微生物例如肠出血性大肠杆菌

(Escherichia coli)、单核细胞增生利斯特菌(Listeria monocytogenes)、艰难梭状芽胞杆菌(Clostridium difficile)、铜绿假单胞菌(Pseudomonas aeruginosa)和沙门氏菌(Salmonella spp.)。

[0030] 本发明的上述用途针对健康个体或有病理学肠道症状的个体。具体地,在健康个体的情况中,本发明组合物在摄取后的个体中产生维持微生物群的稳态的作用和/或预防其改变的作用,并因此其可称为益生菌组合物(或益生菌)。

[0031] 本发明的其他方面涉及组合物的医药用途,所述组合物包含微生物,优选至少一种乳杆菌属副干酪种的细菌,用于治疗和/或预防肠道丁酸盐-依赖性和/或琥珀酸盐-依赖性病理状况。

[0032] 本文中,肠道丁酸盐-依赖性和/或琥珀酸盐-依赖性病理状况表示对用丁酸和/或其盐治疗和/或用琥珀酸和/或其盐治疗敏感的病理状况。示例性的病理为:腹泻、肠道炎症、溃疡性结肠炎、胃萎缩、肠憩室、狭窄、阻塞和糖尿病神经病变。

[0033] 本发明的优选实施方式中,组合物包含副干酪乳杆菌DG细菌株系。细菌菌株副干酪乳杆菌DG由SOFAR S.p.A.于05/05/1995在巴黎的巴斯德研究所的国家微生物保藏中心(CNCM)保藏,保藏号为CNCM I--1572。最初,保藏菌株的名称为干酪乳杆菌DG干酪亚种(Lactobacillus casei DG sub.casei)。

[0034] 本发明的其他实施方式中,丁酸和/或其盐、和/或叶酸和/或其盐、和/或烟酸和/或其盐的所述直接和/或间接肠道生产增加和/或琥珀酸的所述直接和/或间接肠道生产降低归因于肠道微生物群,优选归因于粪球菌属和/或布劳特氏菌属的细菌。

[0035] 本发明的优选实施方式中,丁酸和/或其盐的所述直接和/或间接肠道生产增加归因于粪球菌属的细菌,和/或琥珀酸的所述直接和/或间接肠道生产降低归因于布劳特氏菌属的细菌。

[0036] 因此,包含微生物(优选至少一种乳杆菌属副干酪种的细菌,更优选副干酪乳杆菌DG细菌株系)的组合物可用于改良肠道微生物群中粪球菌属和/或布劳特氏菌属的细菌群的密度,优选从而诱导粪球菌属细菌群的增加和/或布劳特氏菌属细菌群的降低。换句话说,摄入本发明组合物改良肠道微生物群中粪球菌属和/或布劳特氏菌属细菌的含量。具体地,给予所述组合物后粪球菌属细菌增加和/或布劳特氏菌属细菌降低。

[0037] 本文所用组合物包含所述微生物,优选所述至少一种乳杆菌属副干酪种的细菌,其为活的或死的形式,作为裂解物或提取物。

[0038] 在本发明的一个实施方式中,所述组合物包含约150亿至300亿个集落形成单位(CFU)的细菌,优选200至250亿个CFU的细菌。

[0039] 优选所述组合物配置为口服给予。具体而言,所述组合物以固体形式配制,优选丸剂、胶囊剂、片剂、粒状粉末、硬胶囊、水溶性颗粒、囊剂或粒料。

[0040] 或者,本发明的组合物被配制为液体形式,例如糖浆或饮品,或者被添加至食物,例如酸奶、乳酪,或果汁。

[0041] 或者,本发明的组合物以能够发挥局部作用的形式配制,例如,灌肠剂。

[0042] 在本发明的另一个实施方式中,所述组合物还包含一般经许可用于生产益生菌和/或药物产品的赋形剂。

[0043] 在本发明的另一个实施方式中,本发明的组合物可富含维生素、微量元素例如锌

和硒，酶，益生元物质，例如，低聚果糖(FOS)、低聚半乳糖(GOS)、菊粉，瓜尔豆胶或其组合。为了本发明的用途，所述组合物优选一天摄取一次，更优选醒来后摄取。

[0044]　或者，其可在晚间摄取，优选晚餐后。

实施例

[0045]　处理

[0046]　对健康个体进行随机、双盲、安慰剂-对照的交叉饮食干预研究。

[0047]　志愿者按照如下标准征募：

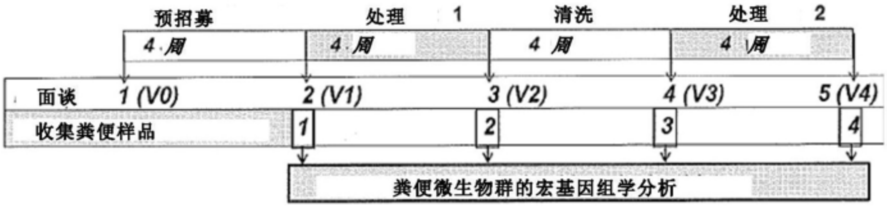
[0048]　-入选标准：健康男性和女性，年龄在18和55岁之间，签署知情同意书；

[0049]　-排除标准：在首次检测之前一个月内有过抗生素处理；在首次检测之前2个月内有病毒或细菌性肠炎事件；在首次检测前5年内有胃溃疡或十二指肠溃疡；妊娠或哺乳期；近期或疑似酒精中毒或药物摄取(吸毒)事件；与该研究方案不相容的其它状况。

[0050]　该益生饮食干预根据交叉设计进行，如下表1所示。

[0051]　表I

[0052]



[0053]　在预登记阶段(4周)，志愿者遵循其常规饮食，不消耗益生发酵的乳品(因此允许传统酸奶)、益生饮食补充剂，或益生元饮食补充剂。

[0054]　在预登记阶段末期，每天随机给予志愿者益生菌或安慰剂的一种胶囊，持续4周。

[0055]　举例而言，采用Enterolactis Plus作为待给予的益生菌；其由包含240亿CFU(集落形成单位)的副干酪乳杆菌DG菌株的420mg胶囊组成。

[0056]　包含安慰剂的胶囊在外观上与包含益生菌的那些相同，其明确地缺乏益生菌。

[0057]　所述活性物质(即益生菌)的味道和颜色与安慰剂相同。

[0058]　该产品在早晨于早餐前至少十分钟空腹摄取，若忘记，则在夜间就寝之前且在任何情况下需于最后一餐之后至少两个小时摄取。

[0059]　在第一个四周处理之后，志愿者经历四周清洗期，与预登记阶段相同。

[0060]　在清洗期终末，志愿者按照上述交叉设计每天摄取Enterolactis Plus或安慰剂的一种胶囊，持续四周。

[0061]　总之，该研究涉及4个阶段，其各自持续4周：

[0062]　•预招募阶段：所述个体既不经历A处理，也不经历B处理。

[0063]　处理1：所述个体经历A处理或B处理。

[0064]　•清洗：所述个体既不经历A处理，也不经历B处理

[0065]　处理2：所述个体经历B处理或A处理。处理A和B可为本发明组合物，具体示例中为Enterolactis plus或安慰剂。处理开始时，不知晓个体摄入的是何物，仅在处理终末期知晓摄取顺序(解除双盲)。

[0066]　检测和样品收集。

[0067] 初始时，指示各志愿者遵循整个过程，这包括与每位志愿者的总计5次会晤。

[0068] 在第一次会晤期间，获得知情同意书以及志愿者的个人数据。并且，告知志愿者所述研究将如何开展的总体信息，并且指示志愿者在预登记的后续4周的饮食上的变化(禁止消耗先前指定的产品)。4周后，志愿者携带粪便样品(T0样品)前来进行第二次会晤，所述粪便样品在先前24小时时程之内收集于第一次会晤交于的特定容器中。

[0069] 为了确保最优保存，将粪便样品贮存在室温，并在24小时之内递送至实验室。

[0070] 此外，在第二次会晤过程中，给予志愿者所述益生产品(或安慰剂)，以供在接下来的4周期间摄取。

[0071] 并且，指示志愿者如何摄取所述产品。

[0072] 在摄取所述产品(或安慰剂)的这4周末期，志愿者携带另一份粪便样品(T1样品)前来进行第三次会晤，所述粪便样品在之前24小时之内收集。

[0073] 在第三次会面过程中，志愿者就源自消耗所述产品的可能效果(积极和不希望的)完成问卷。

[0074] 然后，指示志愿者有关接下来4周的操作，在这期间他或她依然不摄取先前所述的产品。

[0075] 在这4周末期，志愿者携带粪便样品(T2样品)前来进行第四次会面，并且给予其所述益生产品(或安慰剂)以供在接下来4周期间摄取。

[0076] 最后，在摄取所述产品(或安慰剂)4周后，志愿者前来进行第五次会面以递交最后一份粪便样品(T3样品)。

[0077] 在这最后一次会面过程中，志愿者完成与第三次会面中接收到的问卷类似的问卷。

[0078] 在分析微生物群之前，收集的全部粪便样品在-20℃贮存不超过7天。

[0079] 粪便微生物群的分析

[0080] 通过分析编码16S rRNA细菌核糖体亚基的基因的部分的核苷酸序列来评价粪便微生物群。更具体地，采用宏基因组学(metagenomic)策略；简言之，其由如下步骤组成：

[0081] 1.从粪便样品提取宏基因组学DNA，并对其定量和标准化；

[0082] 2.通过PCR扩增编码16S rRNA的细菌基因的V3高变区；

[0083] 3.对PCR产物定量；

[0084] 4.对扩增的产物测序；

[0085] 5.对序列进行生物信息学分析。

[0086] 步骤1和3的操作是本领域熟知的技术，因此，其采用本领域中常用的方案进行。例如，实验室手册中描述的方法，例如见述于Sambrook等.2001或Ausubel等.1994。扩增16S核糖体RNA基因的V3区域的步骤2通过称为PCR的DNA扩增技术进行，采用Probio_Uni 5’-CCTACGGGRSGCAGCAG-3’(SEQ ID NO:1)和Probio_Rev 5’-ATTACCGCGGCTGCT-3’(SEQ ID NO:2)作为寡核苷酸(引物)。

[0087] 具体而言，SEQ ID NO:1和2引物对扩增16S rRNA基因的V3区域。

[0088] 步骤4可采用本领域已知的用于该目的的技术进行，例如基于桑格测序法、焦磷酸测序法或离子激流融合引物测序法的技术，其根据Milani等.(2013)的学术论文的材料与方法部分描述的方案用于本发明的特定实施例中。

[0089] 在离子激流技术的情况中，引物以如下方式设计并合成，以在5’端包括用于该特定DNA测序技术中的两个衔接子序列之一。在该情况中，所述衔接子序列是SEQ ID NO:1和2。

[0090] 进行PCR的条件如下：

[0091] •95℃，5分钟；

[0092] •94℃，30秒；55℃，30秒；和72℃，90秒；进行35个循环；

[0093] •72℃，10分钟。

[0094] 在PCR结束时，通过电泳来检验扩增物的完整性。

[0095] 本方法的步骤5是表征微生物群落所需，可用大量先前已知用于该目的的技术进行。更具体地，利用：分级聚类、分类分析和采用热图构建系统树，按照Milani等.(2013)的学术论文的材料与方法部分中描述的方案；更具体地，序列数据的分析采用QIIME软件进行。

[0096] 数据的统计学分析

[0097] 所述统计学分析采用STATISTICA软件(史丹索特公司(Statsoft Inc.)，美国俄克拉菏马州塔尔萨)进行。

[0098] 为了揭示显著差异，数据采用参数(多变量和单变量重复测试ANOVA)和非参数(沃尔德-沃尔福威茨与曼-惠特尼)统计学方法分析。

[0099] 数据集的正态性(ANOVA的重要假设)通过夏皮洛-威尔克(Shapiro-Wilk)和柯莫果夫-斯米尔诺夫(Kolmogorov-Smirnov)检验评价。

[0100] 处理的结果

[0101] 该研究由总计22位个体(11位女性和11位男性)完成。

[0102] 初始时登记了33位个体，但其中11位因下述原因在早期退出了：摄入抗生素(4)，拒绝继续该研究(1)，频发腹泻(1)，在该研究期间摄入其它益生菌(3)，饮食习惯大幅变化(1)，和，伴有穿插腹泻的季节性流感(1)。

[0103] 在总结研究并完成两次处理的结果分析之后，揭示双盲结果，并且观察到：A处理是包含副干酪乳杆菌DG的活性处理；B处理是外表与活性处理相同的安慰剂，但缺乏乳杆菌。

[0104] 当分析获自该研究的数据时，就分类学而言，观察到该研究参与者的肠道微生物群的高稳定性。

[0105] 事实上，发现：

[0106] a)15种鉴定的细菌中的两种细菌分离物，即，拟杆菌(Bacteroidetes)和厚壁菌(Firmicutes)，占超过90％的序列；

[0107] b)131种鉴定的家族中的11种占超过90％的序列；和

[0108] c)262种鉴定的属中的20个属占超过90％的序列。

[0109] 此外，该研究证实，处于较低分类水平(即，处于家族和属水平)的人类肠道微生物群在个体彼此之间高度可变。

[0110] 因此，实验证据显示，对健康群体进行交叉干预试验的必要性，以防止显著的个体间变异性会隐藏益生菌处理的可能作用或导致统计学假阳性。当评价由两种处理诱导的肠道微生物群的变化时，仅在接受副干酪乳杆菌DG处理(活性处理)的组中出现了以属为单元

的统计学显著差异。更具体地，观察到粪球菌属的增加。事实上，由图1.1、2.1和2.2可见，在用副干酪乳杆菌DG处理之前和之后，观察到粪球菌的统计学显著增加。相反，在接受安慰剂处理的组中观察到中度减少。

[0111] 此外，用副干酪乳杆菌DG处理之后，观察到布劳特氏菌属的细菌的统计学显著减少。相反，在接受安慰剂处理的组中观察到其轻微增加(图1.2、2.1和2.2)。

[0112] 粪球菌是肠道水平的主要丁酸盐生成者之一。

[0113] 丁酸盐是肠道水平的基础化合物，因为，一方面，其有助于恢复肠道粘膜的功能完整性并随时间进展维持该状况，而在另一方面，其具有重要的抗炎作用，以至于其被用作肠道结肠病(例如慢性炎性肠道疾病)的饮食处理的佐剂。

[0114] 此外，其基因组的分析揭示，这些细菌能够利用琥珀酸盐作为发酵底物。

[0115] 以该信息为基础，考虑到布劳特氏菌属的成员产生乙酸盐和琥珀酸盐作为葡萄糖发酵的主要终产物。

[0116] 考虑琥珀酸盐是产生溃疡的因素，因此，能够使患有溃疡性结肠炎的个体状况恶化，因为其在上述疾病的活跃期间很可能造成粘膜损伤。

[0117] 因此，在采用益生菌处理之后，在该情况中，在给予副干酪乳杆菌DG之后，观察到属于粪球菌属的细菌的增加，以及进而的，肠道丁酸盐浓度的增加。

[0118] 同时，观察到可能造成患有溃疡性结肠炎的个体的粘膜损伤的琥珀酸盐的浓度直接下降，因为在用益生菌处理之后(在本文中为给予了副干酪乳杆菌DG之后)属于布劳特氏菌属的细菌间接地有所减少，这是因为增加的粪球菌群还能够通过以琥珀酸盐作为其发酵加工的底物而降低琥珀酸盐浓度。

[0119] 因此，在用益生菌处理之后，在具体实施例中为给予副干酪乳杆菌DG之后，个体粪便中的丁酸浓度增加，同时其它有机酸(例如琥珀酸)减少。

[0120] 最后，在以基于细菌基因组的认知进行宏基因组学的虚拟重建为目的的生物信息学分析中，采用与粪便微生物群的组成相关的数据(Okuda S，Tsuchiya Y，Kiriyama C，Itoh M，Morisaki H.“16S rRNA基因序列的虚拟宏基因组重建(Virtual metagenome reconstruction from 16S rRNA gene sequences)”.Nat Commun.2012;3:1203)；换言之，其以电脑模拟方式建立存在何种可能的基因和给定微生物群的丰度如何。该分析使得检验供于叶酸的合成和烟酸的代谢(图3和4)的编码基因的推定增加成为可能。这两种分子是对于人宿主而言重要的维生素(分别称为维生素B9和B3)。具体而言，维生素B9代表首要的营养因子，缺乏它(尤其是在特殊生理状况，例如妊娠中)会导致严重的健康后果。因此，采用本研究所用益生菌进行处理能够促进肠道微生物群生成叶酸(维生素B9)的能力，这对人类宿主具有后续营养益处。

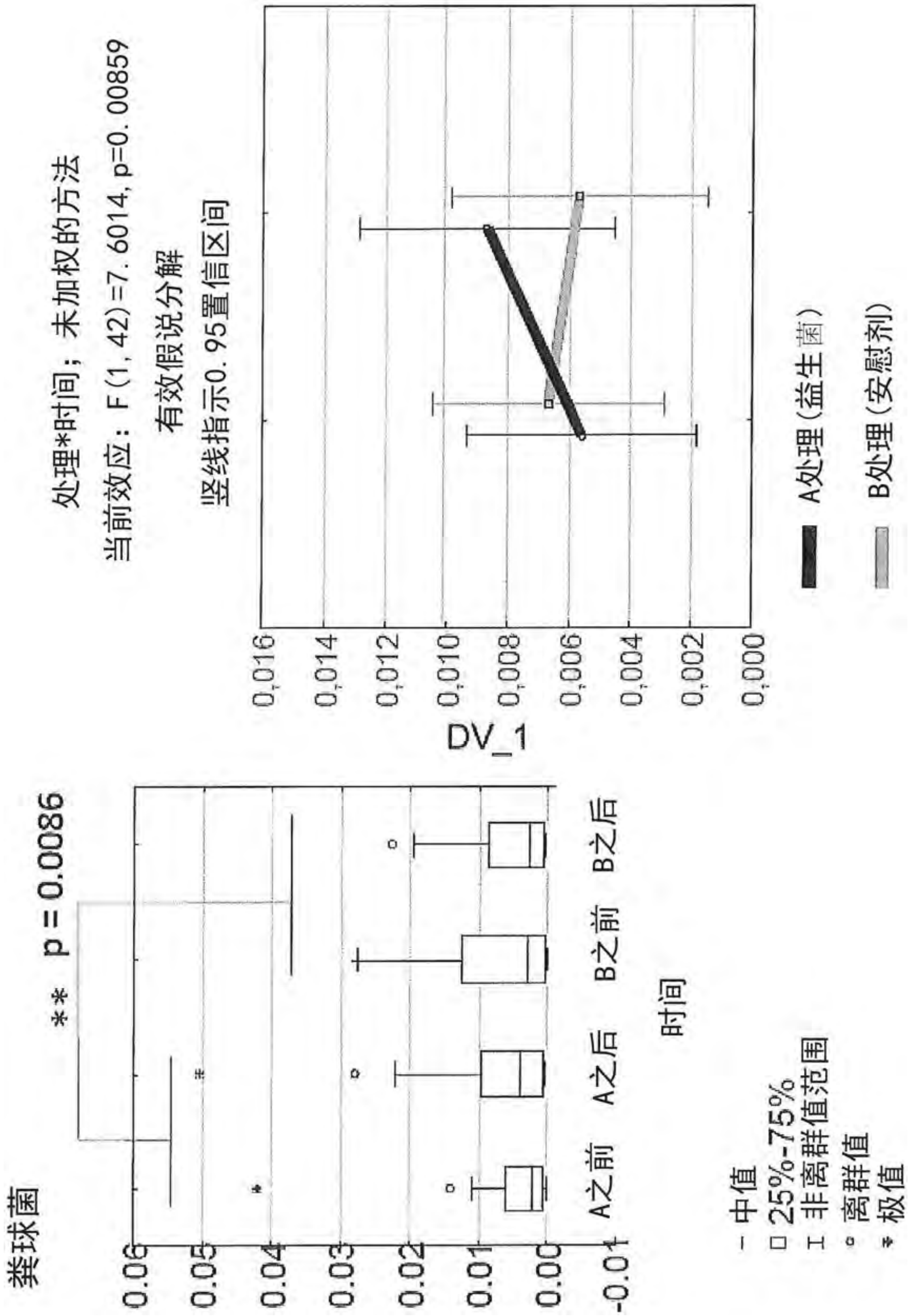

粪球菌
** p = 0.0086
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
A之前
A之后
B之前
B之后
时间
− 中值
□ 25%-75%
I 非离群值范围
○ 离群值
* 极值
处理*时间；未加权的方法
当前效应：F(1, 42)=7.6014, p=0.00859
有效假说分解
竖线指示0.95置信区间
DV_1
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
A处理(益生菌)
B处理(安慰剂)

图1.1

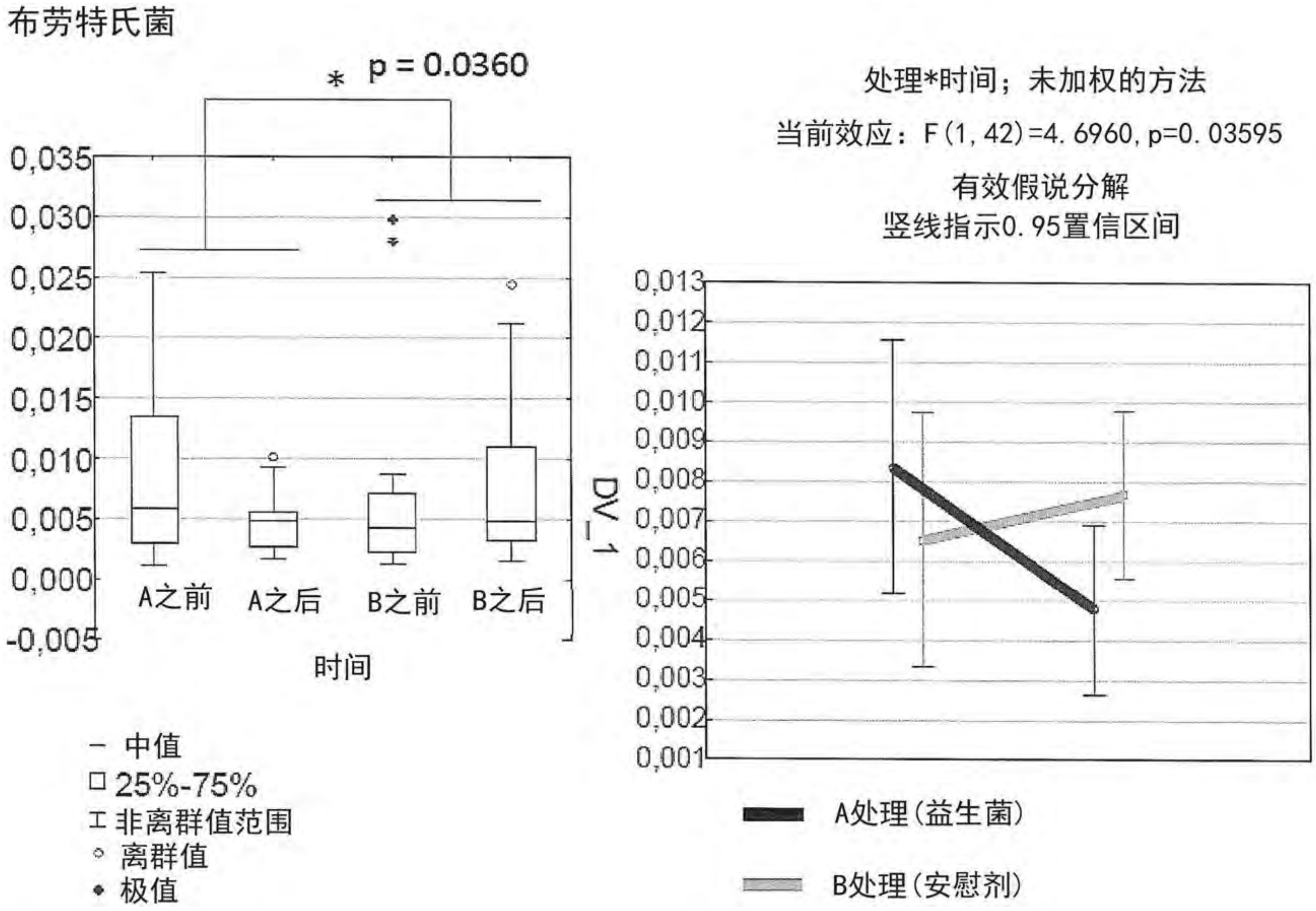

布劳特氏菌
* p = 0.0360
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
-0,005
A之前
A之后
B之前
B之后
时间
DV_1
– 中值
□ 25%-75%
⌶ 非离群值范围
○ 离群值
◆ 极值
处理*时间；未加权的方法
当前效应：F(1, 42)=4.6960, p=0.03595
有效假说分解
竖线指示0.95置信区间
0,013
0,012
0,011
0,010
0,009
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
A处理(益生菌)
B处理(安慰剂)

图1.2

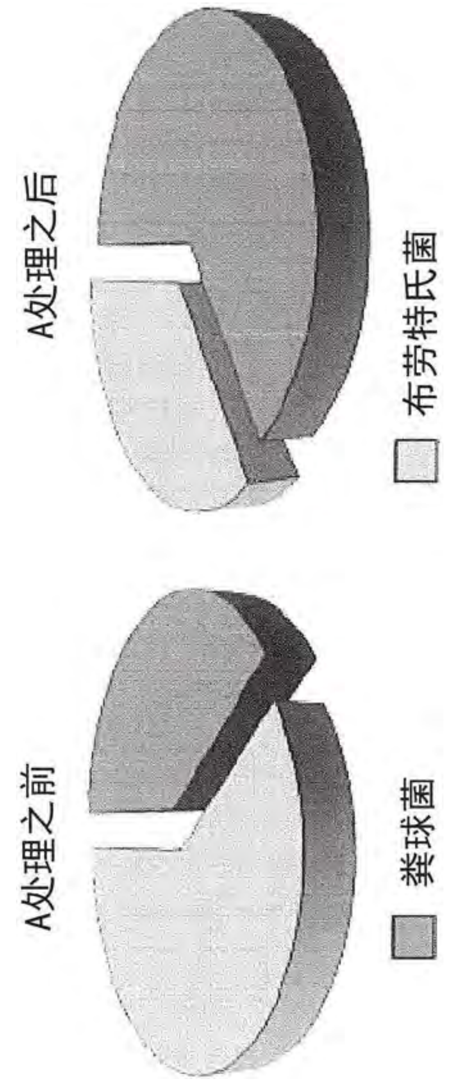


图2.1

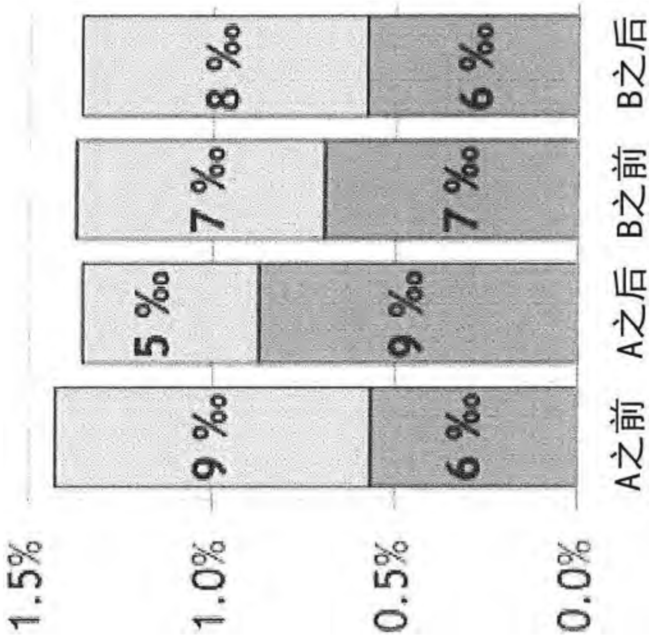


图2.2

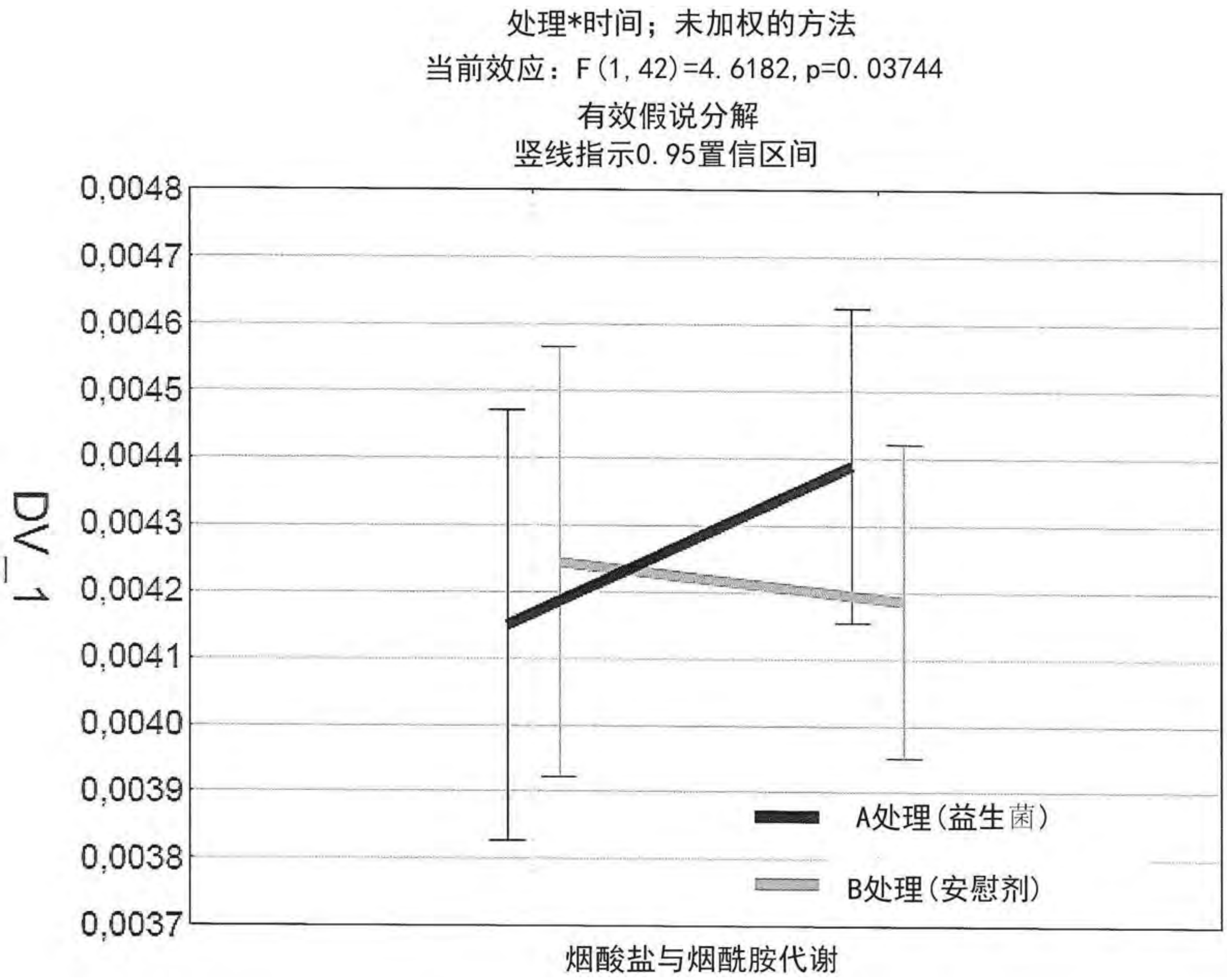

处理*时间；未加权的方法
当前效应：F(1, 42)=4.6182, p=0.03744
有效假说分解
竖线指示0.95置信区间
DV_1
0,0048
0,0047
0,0046
0,0045
0,0044
0,0043
0,0042
0,0041
0,0040
0,0039
0,0038
0,0037
A处理(益生菌)
B处理(安慰剂)
烟酸盐与烟酰胺代谢

图3

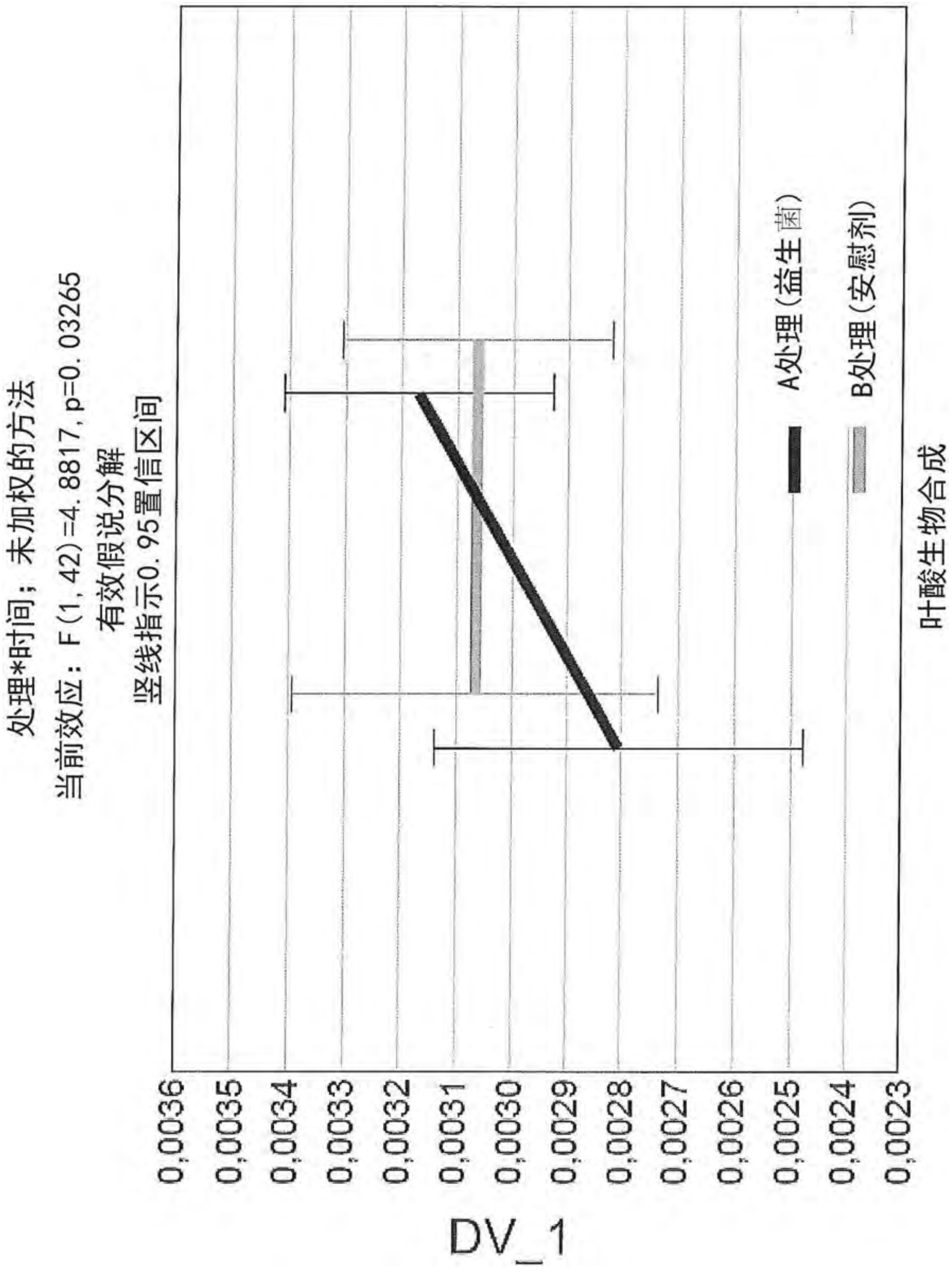

处理*时间；未加权的方法
当前效应：F(1，42)=4.8817，p=0.03265
有效假说分解
竖线指示0.95置信区间
DV_1
0,0036
0,0035
0,0034
0,0033
0,0032
0,0031
0,0030
0,0029
0,0028
0,0027
0,0026
0,0025
0,0024
0,0023
A处理(益生菌)
B处理(安慰剂)
叶酸生物合成

图4