(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 March 2015 (12.03.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/033304 A1

(51) International Patent Classification:
C12Q 1/10 (2006.01) *C12Q 1/68* (2006.01)
A23L 1/30 (2006.01) *C12R 1/225* (2006.01)
A61K 35/74 (2015.01)

(21) International Application Number:
PCT/IB2014/064284

(22) International Filing Date:
5 September 2014 (05.09.2014)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
MI2013A001473 6 September 2013 (06.09.2013) IT

(71) Applicant: **SOFAR S.P.A.** [IT/IT]; Via Firenze 40, I-20060 Trezzano Rosa (milano) (IT).

(72) Inventors: **BIFFI, Andrea**; Via Mulino Vecchio 146, I-24059 Urgnano (bergamo) (IT). **ROSSI, Ruggero**; Via Federico Engels 1, I-20153 Milano (IT). **FIORE, Walter**; Via Giuseppe Rossini 12, I-20060 Trezzano Rosa (milano) (IT). **GUGLIELMETTI, Simone Domenico**; Via Cima Otto e Camillo 23, I-20134 Milano (IT).

(74) Agents: **ERRICO, Michela** et al.; c/o Bugnion S.P.A., Viale Lancetti 17, I-20158 Milano (IT).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: METHOD FOR EVALUATING THE EFFECTS OF A COMPOSITION COMPRISING MICROORGANISMS ON INTESTINAL MICROBIOTA

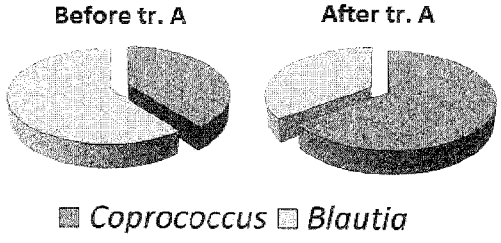


Fig.2.1

(57) Abstract: The present invention relates to a method for determining the probiotic/paraprobiotic activity of a composition comprising microorganisms, in particular bacteria, said method being based on evaluating, by metagenomic analysis, the qualitative and/or quantitative change in faecal microbiota following intake of the composition. Moreover, the present invention relates to a kit for carrying out said method.

WO 2015/033304 A1

DESCRIPTION

"METHOD FOR EVALUATING THE EFFECTS OF A COMPOSITION COMPRISING MICROORGANISMS ON INTESTINAL MICROBIOTA"

The present invention relates to a method for determining the probiotic/paraprobiotic activity of a composition comprising microorganisms, in particular bacteria, said method being based on an evaluation of the qualitative and/or quantitative change in faecal microbiota following intake of the composition. Moreover, the present invention relates to a kit for carrying out said method.

The gastrointestinal tract comprises numerous populations of microorganisms which have developed and multiplied during the development of each individual and form the so-called intestinal microbiota or intestinal flora.

Therefore, the intestinal microbiota represents a highly complex ecosystem and the condition of equilibrium among the different populations of microorganisms making it up, or so-called eubiosis, 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 broad spectrum of pathologies.

For the purpose of treating a condition of intestinal disbiosis, or in any case for the purpose of maintaining the equilibrium of the intestinal microbiota, the practice of taking probiotic/paraprobiotic products is becoming more and more frequent.

According to the definition of the FAO/WHO, a probiotic is a set of "live microorganisms which, when administered in adequate amounts, confer a

health benefit on the host".

In light of the above, the advantages tied to the development of a method making it possible to evaluate, quickly and reliably, the effects of an exogenous composition/formulation comprising microorganisms on the bacterial composition of the intestinal microbiota of an individual are fairly evident.

In fact, on the basis of the effects measured with such a method, i.e. on the basis of how the intake of the composition comprising microorganisms quantitatively and/or qualitatively modifies the intestinal microbiota, it will be possible to establish whether said composition is capable of favouring and/or ensuring the well-being and health of the human body and, therefore, whether it fulfils one of the fundamental prerequisites for being identified as a probiotic/paraprobiotic.

The present invention fulfills the above-mentioned requirements by providing a method for determining, by molecular analysis, the qualitative and/or quantitative change in the composition of the faecal microbiota of an individual following intake of a composition comprising microorganisms, preferably bacteria, according to a randomized, double-blind, placebo-controlled crossover protocol.

In fact, the Applicant has experimentally demonstrated, for the very first time, the necessity of conducting crossover intervention study protocols, especially on a healthy population, in order to prevent the marked inter-individual variability from hiding the possible effects of a treatment, in particular a treatment with a probiotic/paraprobiotic, or from leading to false statistical positives.

The method of the present invention, besides being particularly advantageous for the purpose of determining the effects of a generic composition comprising microorganisms (i.e. a presumed probiotic/paraprobiotic) on faecal microbiota, is also useful for the purpose of confirming the health-promoting effect of a known probiotic/paraprobiotic on the human body, or for the purpose of

determining any new specific effects of a known probiotic/paraprobiotic, for example by studying which populations of microorganisms are stimulated and/or inhibited in their growth following intake of the composition. In fact, on the basis of the main activities in which the populations of microorganisms whose growth is stimulated and/or inhibited following intake of the composition are involved, it will be possible to define the possible new effects of the same. For example, if, following intake of a probiotic according to the method of the present invention, it is found that a particular bacterial population has grown in quantitative terms and that this bacterial population has a metabolism mainly involved in the production, for example of butyric acid, it can be deduced that the probiotic can be taken in order to increase the amount of butyric acid in the intestinal tract.

Further advantages of the method 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 shows the result of the statistical analysis conducted in order to evaluate the increase in the population of bacteria of the genus *Coprococcus* (Fig.1.1) and the decrease in the population of bacteria of the genus *Blautia* (Fig.1.2) before and after treatment with the composition of the present invention (A) and, at same time, the decrease in the population of bacteria of the genus *Coprococcus* (Fig.1.1) and the increase in the population of bacteria of the genus *Blautia* (Fig.1.2) 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 conducted to establish the increase in the metabolism of nicotinic acid before and after treatment with the composition of the present invention and the decrease therein before and after treatment with the placebo; and
- Figure 4 shows the result of the statistical analysis conducted to establish 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.

A first aspect of the present invention relates to a method for determining the change in the composition of the faecal microbiota of an individual following intake of a composition/formulation comprising microorganisms, according to a randomized, double-blind, placebo-controlled crossover protocol, said method comprising the steps of:

a) collecting information about the state of health and/or the eating habits of said individual before and/or during and/or after taking the composition or placebo according to a randomized, double-blind placebo-controlled crossover protocol;

b) obtaining at least one faecal sample from the individual before and/or during and/or after intake of the composition or placebo according to a randomized, double-blind placebo-controlled crossover protocol;

c) analyzing the microbiota by metagenomic analysis conducted on the faecal sample obtained in step b);

d) comparing, preferably qualitatively and/or quantitatively, the faecal microbiota of the individual before and/or during and/or after intake of the composition or placebo according to a randomized, double-blind, placebo-controlled crossover protocol.

In the context of the present invention, the term faecal microbiota means the whole of the populations of microorganisms which are present within the faeces of an individual and reflect the whole of the populations of microorganisms present in the intestine of the same. Therefore, the term faecal microbiota is meant here as a synonym of intestinal microbiota.

In particular, the microorganisms included in the composition of the present invention are bacteria and/or yeasts and/or other microorganisms, taken individually or in combination.

A composition comprising bacteria is particularly preferred for the purposes of the present invention. In particular, the bacteria belong to the genus selected from: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Enterococcus*. More preferably, said bacterium is of the genus *Lactobacillus* and/or *Bifidobacterium*.

In particular, the *Lactobacillus* is selected from: *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *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 kefiri*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus collinoides*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus*

plantarum, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius* and *Lactobacillus sanfranciscensis.*

Particularly preferred for the purposes of the present invention are bacteria belonging to the species *Lactobacillus paracasei*, more preferably the 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 strain had the denomination of Lactobacillus *casei* DG *sub.casei.*

In particular, the bacteria of the genus *Bifidobacterium* are selected from: *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum.*

The yeasts are preferably of the genus *Saccharomyces*, more preferably of the species *Saccharomyces cerevisiae*.

In general, the microorganisms included in the composition of the present invention are individual microorganisms or combinations of any microbial species specified in the QPS list of the EFSA (http://www.efsa.europa.eu/it/search/doc/3020.pdf).

The microorganisms of the composition of the present invention are preferably live and the composition is thus also definable as a probiotic. Alternatively, the microorganisms of the composition are dead and/or in the form of a lysate or extract and hence the composition is also definable as a paraprobiotic. Therefore, the composition of the present invention is also a known or presumed probiotic or paraprobiotic.

In one embodiment of the invention, the composition comprises about 1-50 billion colony forming units (CFU) of microorganisms, preferably 15-30, more preferably 20-25 billion CFU of microorganisms.

In one embodiment of the present invention, the composition is formulated for oral administration. In particular, the composition is formulated in solid

form, preferably as pills, capsules, tablets, granular powder, hard capsules, water-soluble granules, sachets or pellets.

Alternatively, the composition of the invention is formulated as a liquid, for example as a syrup or beverage, or else is added to a food, for example 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 a further embodiment of the invention, the composition also 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 is enriched with vitamins, trace elements such as zinc and selenium, enzymes and/or prebiotic substances such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, guar gum or combinations thereof.

As regards intake of the composition, as earlier explained, it follows a randomized, double-blind, placebo-controlled crossover protocol. In other words, during intake neither the investigator nor the individuals included in the trial are aware of the assigned treatments (the treatments are indistinguishable, double blind) and a same individual is exposed at different times to treatment both with the composition containing microorganisms and with the placebo (crossover), according to a random sequence.

In one embodiment of the invention, said protocol comprises the following phases: 1) a pre-recruitment phase, in which the individuals preferably do not take the composition comprising microorganisms or the placebo; and/or 2) a first treatment phase, in which the individuals preferably take the composition comprising microorganisms or the placebo; and/or 3) a wash-out phase, in which the individuals preferably do not take the composition comprising microorganisms or the placebo; and/or 4) a second treatment phase, in which the individuals preferably take the

placebo or the composition comprising microorganisms.

Intake as per phase 2 and phase 4 takes place in a random, double-blind manner as specified above. It is clear that the individual who takes the placebo in the first phase will take the composition comprising microorganisms in the second phase and vice versa.

The duration of the different phase of the protocol is preferably the same. In particular, the duration of at least one of these phases, preferably of all the phases, is about four weeks.

In the context of the present invention, the term wash-out means a period falling between two phases of taking the composition comprising microorganisms or a placebo in which the individual does not take anything and should thus "expel" what he or she has taken previously, i.e. a period of absence of treatment aimed at eliminating every residual effect.

In one embodiment of the invention, the composition of the present invention is taken preferably once a day, more preferably right after awakening.

Alternatively, taking it in the evening is also possible, preferably at least 3 hours after meals.

The step of collecting information regarding the state of health and/or the eating habits of the individual is preferably carried out by gathering said information in a questionnaire. Said questionnaire is prepared *ad hoc* to collect data regarding the state of health and/or the eating habits of an individual who implements the method of the invention.

In particular, said questionnaire is a standard sheet on which questions related to the state of health and/or the eating habits of said individual are formulated. As regards the state of health, the individual can respond using rating scales associated with each question. The rating scale is preferably a verbal numerical scale (VNS), or a visual analogue scale (VAS) or verbal rating scale (VRS). As regards eating habits, the individual can respond by indicating the foods he or she consumes daily, also specifying the amounts consumed where possible.

The step of collecting information is preferably carried out at the start of the pre-recruitment phase and/or before and/or after the first treatment phase and/or before and/or after the end of the second treatment.

The obtainment of at least one faecal sample preferably takes place at the start and/or at the end of the first treatment and/or at the start and/or at the end of the second treatment.

The faecal sample is preferably taken no earlier than 48 hours before, more preferably no earlier than 24 hours before being processed or stored at a temperature preferably comprised between +4°C and -20°C, more preferably at -20°C, for a period that preferably does not exceed 7-10 days. Storage of the faecal sample before processing or storage at a low temperature preferably takes place at room temperature.

The step of analyzing the microbiota by metagenomic analysis of the faecal sample is carried out on the nucleic acids, preferably on the DNA extracted from the faecal microbiota.

In particular, the analysis of the microbiota by metagenomic analysis comprises at least one, and preferably all, of the following steps:

- extracting the nucleic acids, preferably of the DNA from the faecal sample; and
- molecularly typing the faecal microbiota.

Extraction of the nucleic acids in general, and DNA in particular, from the faecal sample is achieved using the procedures known to every person skilled in the art for that purpose.

In one embodiment of the invention, the typing of populations of microorganisms is achieved by analyzing the nucleotide sequence of at least one portion of the gene encoding a subunit of the ribosome, preferably the 16S subunit of the ribosome, i.e. the gene encoding the 16S rRNA molecule.

For this purpose, the DNA extracted from the faecal samples is amplified using techniques known in the art, for example by PCR. Preferably, the amplification is achieved by using a pair of oligonucleotides (primers);

preferably by using SEQ ID NO: 1 (Probio_Uni 5'-CCTACGGGRSGCAGCAG-3') and SEQ ID NO: 2 (Probio_Rev 5'-ATTACCGCGGCTGCT-3') (Milani C, Hevia A, Foroni E, Duranti S, Turroni F, et al. (2013) Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. PLoS ONE 8(7): e68739).

The conditions for carrying out the PCR can vary depending on the quality and quantity of the nucleic acid it is desired to amplify and/or the primers used. In any case, setting the PCR conditions is a routine activity for every person skilled in the art.

Preferably, the portions of amplified nucleic acid are subsequently sequenced.

The person skilled in the art can use any known method for that purpose. Preferably, the methods used are selected from: sequencing based on the Sanger method, pyrosequencing methods and the Ion Torrent sequencing method.

In the case of Ion Torrent, it is preferable to use primers that preferably have adaptor sequences at the 5' end. In the particularly preferred embodiment of the present invention, the adaptor sequences are SEQ ID NO: 1 and 2.

Once the sequences have been obtained and, therefore, once the populations of microorganisms of the faecal microbiota have been typed, the community of microorganisms is characterized, preferably by means of hierarchical clustering programs or taxonomic analysis and/or by constructing phylogenetic dendrograms, preferably with heat maps. To this end, QIIME software is particularly preferred for the purposes of the present invention.

Finally, the data obtained from the characterization analyses are preferably analyzed with statistical methods of a parametric and/or non-parametric type.

A further aspect of the present invention regards a kit for performing the method according to the present invention, said kit comprising:

- an identification code of the kit;
- at least one oral formulation of a composition comprising microorganisms, preferably belonging to the species *Lactobacillus paracasei*, more preferably the strain *Lactobacillus paracasei* DG, in an amount of between 1 and 50 billion colony forming units (CFU) of microorganisms, preferably 15-30, more preferably 20-25 billion CFU of microorganisms.
- at least one oral formulation of a placebo not containing microorganisms;

said composition of microorganisms being taken according to a randomized, double-blind crossover protocol controlled vis-à-vis said placebo, and said composition comprising microorganisms and said placebo being identified by a code.

The placebo is preferably identical in aesthetic appearance, i.e. in form, but differs in substance from said oral formulation of a composition comprising microorganisms, preferably belonging to the species *Lactobacillus paracasei*, more preferably the strain *Lactobacillus paracasei* DG. The oral formulation of the placebo contains no microorganisms.

In one preferred embodiment of the invention, the kit comprises at least 28 capsules or tablets or pills or buccal tablets or hard capsules or sachets containing the oral formulation of the composition comprising microorganisms, and, preferably, an equal number of tablets or pills or buccal tablets or hard capsules or sachets containing the oral formulation of placebo.

According to a preferred embodiment of the invention, said at least one oral formulation is at least one capsule, at least one tablet, at least one pill, at least one buccal tablet, at least one hard capsule, at least one sachet or at least one pellet.

Said oral formulations are identified by a code, for example a colour code, a numerical code, an alphabetic code, or an alphanumeric code. For the purpose of the method, said code will serve to understand when the composition comprising microorganisms has been taken and when the

placebo as earlier described has been taken.

The oral formulations are identical in aesthetic appearance, i.e. in form, but differ in substance because one contains the composition comprising microorganisms and the other one contains a placebo. Moreover, the two formulations are each identified by a code.

In this manner, the composition comprising microorganisms and the placebo contained within a kit are such as to be indistinguishable by any individual. Moreover, the composition comprising microorganisms and the placebo contained in the kit are univocally identified by any code whatsoever, for example a colour code, a numerical code, an alphabetic code, or an alphanumeric code.

The correspondence of this code with the nature of the substance, i.e. whether it is the composition comprising microorganisms or the placebo, is known only to the producer of the kit.

According to a further embodiment of the present invention, the kit further comprises questionnaires prepared *ad hoc* for collecting data regarding the state of health of the individual who implements the method of the invention.

In particular, the questionnaires are standard sheets on which questions related to the state of health and/or the eating habits of said individual are formulated. As regards the state of health, the individual can respond using rating scales associated with each question. The rating scale is preferably a verbal numerical scale (VNS), or a visual analogue scale (VAS) or verbal rating scale (VRS). As regards eating habits, the individual can respond by indicating the foods he or she consumes daily, also specifying the amounts consumed where possible.

A further aspect of the present invention regards the use of said kit for diagnostic and/or therapeutic purposes.

EXAMPLE

Treatment.

A randomized, double-blind, placebo-controlled crossover study of dietary

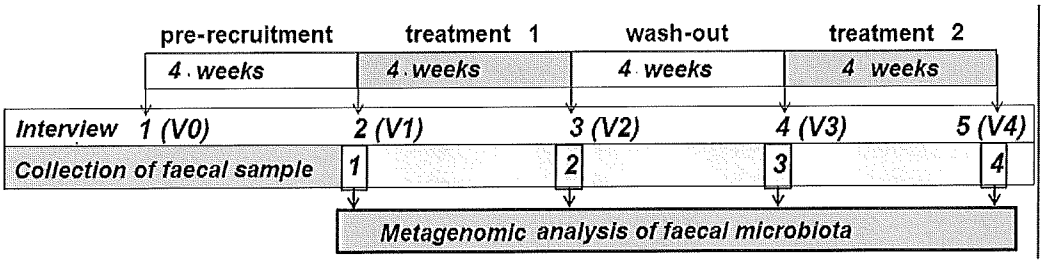
intervention was conducted on healthy individuals.

Volunteers were recruited in accordance with the following criteria:

- inclusion criteria: healthy men and women, ranging in age between 18 and 55 years; signing of informed consent form;
- exclusion criteria: antibiotic treatment in the month preceding the 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 crossover design, as schematized in Table I below.

Table I



In the pre-enrolment step (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 with Enterolactis Plus, nor treatment with the placebo.
- *Treatment 1*: the individuals underwent treatment with Enterolactis Plus or treatment with the placebo.
- *Wash-out*: the individuals underwent neither treatment with Enterolactis Plus, nor treatment with the placebo.
- *Treatment 2*: the individuals underwent treatment with the placebo or treatment with Enterolactis Plus, respectively.

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 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 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:

a) two bacterial divisions of the 15 identified, namely, Bacteroidetes and Firmicutes, constitute over 90% of the sequences;

b) 11 families of the 131 identified constitute over 90% of the sequences; and

c) 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 seen 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 favour the ability of intestinal microbiota to produce folic acid (vitamin B9), with a consequent nutritional benefit for the human host.

CLAIMS

1. An *in vitro* method for determining the change in the composition of the faecal microbiota of an individual following intake of a composition comprising microorganisms and a placebo, according to a randomized, double-blind, placebo-controlled crossover protocol said method comprising the steps of:
   (i) collecting information about the state of health and/or the eating habits of said individual before and/or during and/or after taking the composition or placebo according to said protocol;
   (ii) obtaining a faecal sample from the individual before and/or during and/or after intake of the composition or placebo according to said protocol;
   (iii) analyzing the microbiota by performing a metagenomic analysis on the faecal sample obtained according to step (ii);
   (iv) comparing, preferably qualitatively and/or quantitatively, the faecal microbiota of the individual before and/or during and/or after taking the composition or placebo according to said protocol.
2. The method according to claim 1, wherein said microorganisms are bacteria and/or yeasts taken individually or in combination.
3. The method according to claim 1 or 2, wherein said microorganisms belong to the genus selected from: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Enterococcus*; preferably said microorganism is a bacterium of the genus *Lactobacillus* and/or *Bifidobacterium*.
4. The method according to any one of claims 1-3, wherein said microorganisms are bacteria of the genus *Lactobacillus* selected from the species: *Lactobacillus paracasei, Lactobacillus acidophilus, 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 kefiri, Lactobacillus mucosae, Lactobacillus panis, Lactobacillus collinoides, Lactobacillus paraplantarum, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus pontis, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus sakei, Lactobacillus salivarius and *Lactobacillus sanfranciscensis.*

5. The method according to any one of claims 1-4, wherein said microorganisms are bacteria of the species *Lactobacillus paracasei*, more preferably it is the strain *Lactobacillus paracasei* DG.
6. The method according to any one of claims 1-5, wherein said microorganisms are present in the composition in an amount of between 1 and 50 billion colony forming units (CFU) of microorganisms, preferably 15-30, more preferably 20 to 25 billion CFU of microorganisms.
7. The method according to any one of claims 1-6, wherein said microorganisms are present in the composition as live or dead microorganisms, or in the form of a lysate or extract.
8. The method according to any one of claims 1-7, wherein the composition comprising microorganisms is formulated for oral administration, preferably in solid form, preferably as in the form of pills, capsules, tablets, granular powder, hard capsules, water-soluble granules, sachets or pellets.
9. The method according to any one of claims 1-8, wherein the metagenomic analysis comprises at least one, preferably all, of the following steps:

- Extracting the nucleic acids, preferably DNA, from the faecal sample, and
- Molecularly typing the microorganisms present in the faecal microbiota.

10. The method according to claim 9, wherein the typing of faecal microbiota is performed by analyzing the nucleotide sequence of at least a portion of the gene encoding the 16S subunit of the ribosome.

11. The method according to claim 9 or 10, wherein the typing of faecal microbiota is achieved by amplifying the nucleotide sequence of at least a portion of the gene encoding the 16S subunit of the ribosome by PCR.

12. The method according to claim 11, wherein the PCR is performed using SEQ ID NO: 1 and 2.

13. The method according to claim 11 or 12, wherein the amplified nucleotide sequence is sequenced, preferably using the technique of Ion Torrent sequencing.

14. The method according to any one of claims 9-13, wherein the microorganisms are characterized by means of hierarchical clustering programs and/or taxonomic analysis, and/or by constructing phylogenetic dendrograms, preferably with heat maps.

15. The method according to claim 14, wherein the results of the characterization are analyzed by using parametric and/or nonparametric statistical methods.

16. A kit for performing the method according to any one of claims 1-15 comprising:

- an identification code of the kit;
- at least one oral formulation of a composition comprising microorganisms, preferably of the species *Lactobacillus paracasei*, more preferably the strain *Lactobacillus paracasei* DG, in an amount of between 1 and 50 billion colony forming

units (CFU) of microorganisms, preferably 15-30, more preferably 20 to 25 billion CFU of microorganisms;

- at least one oral formulation of a placebo not containing microorganisms;

said composition of microorganisms being taken according to a randomized, double-blind crossover protocol controlled vis-à-vis said placebo, and said composition comprising said microorganisms and placebo being identified by a code.

Fig.1.1

Coprococcus ssp.

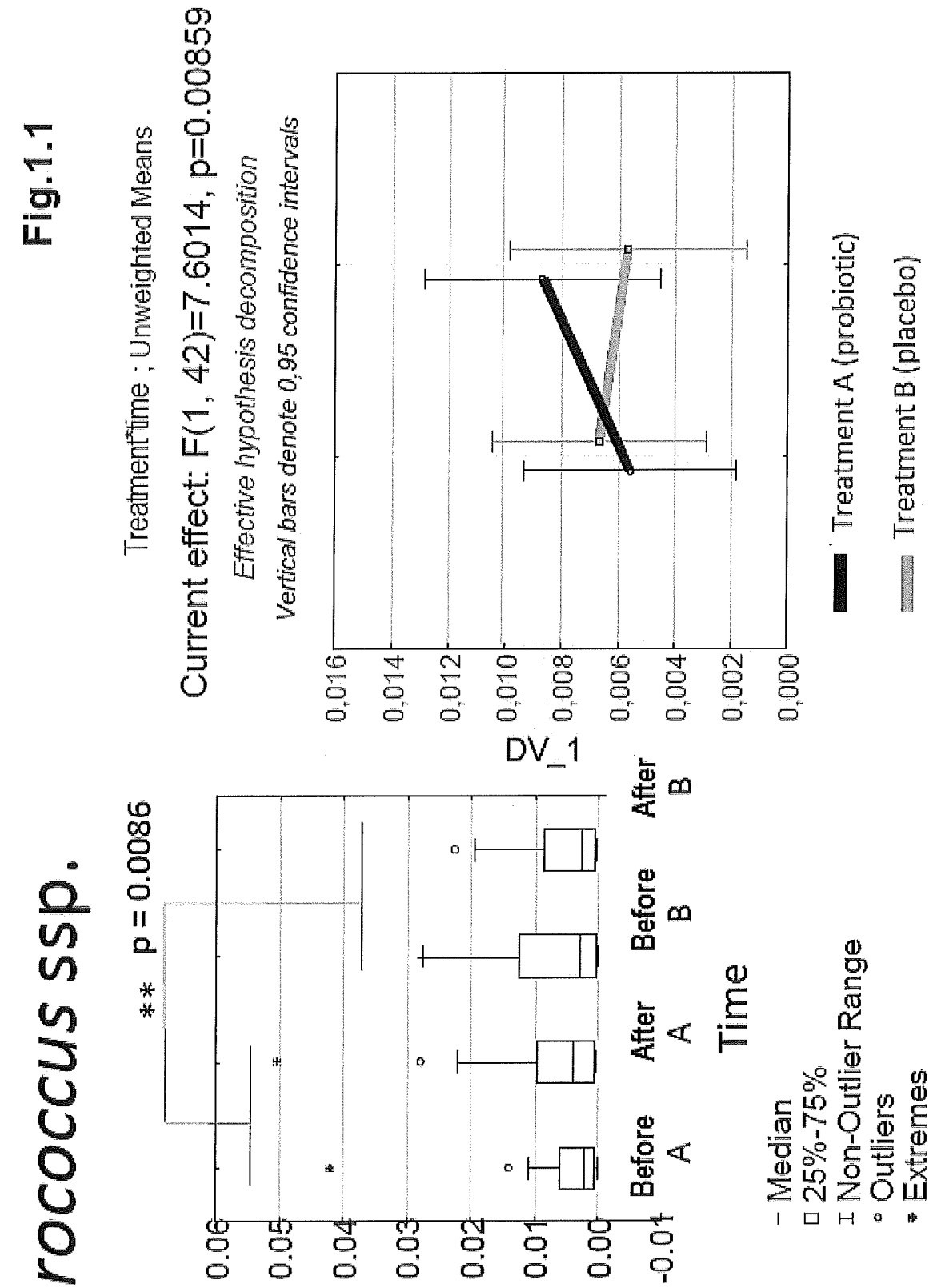
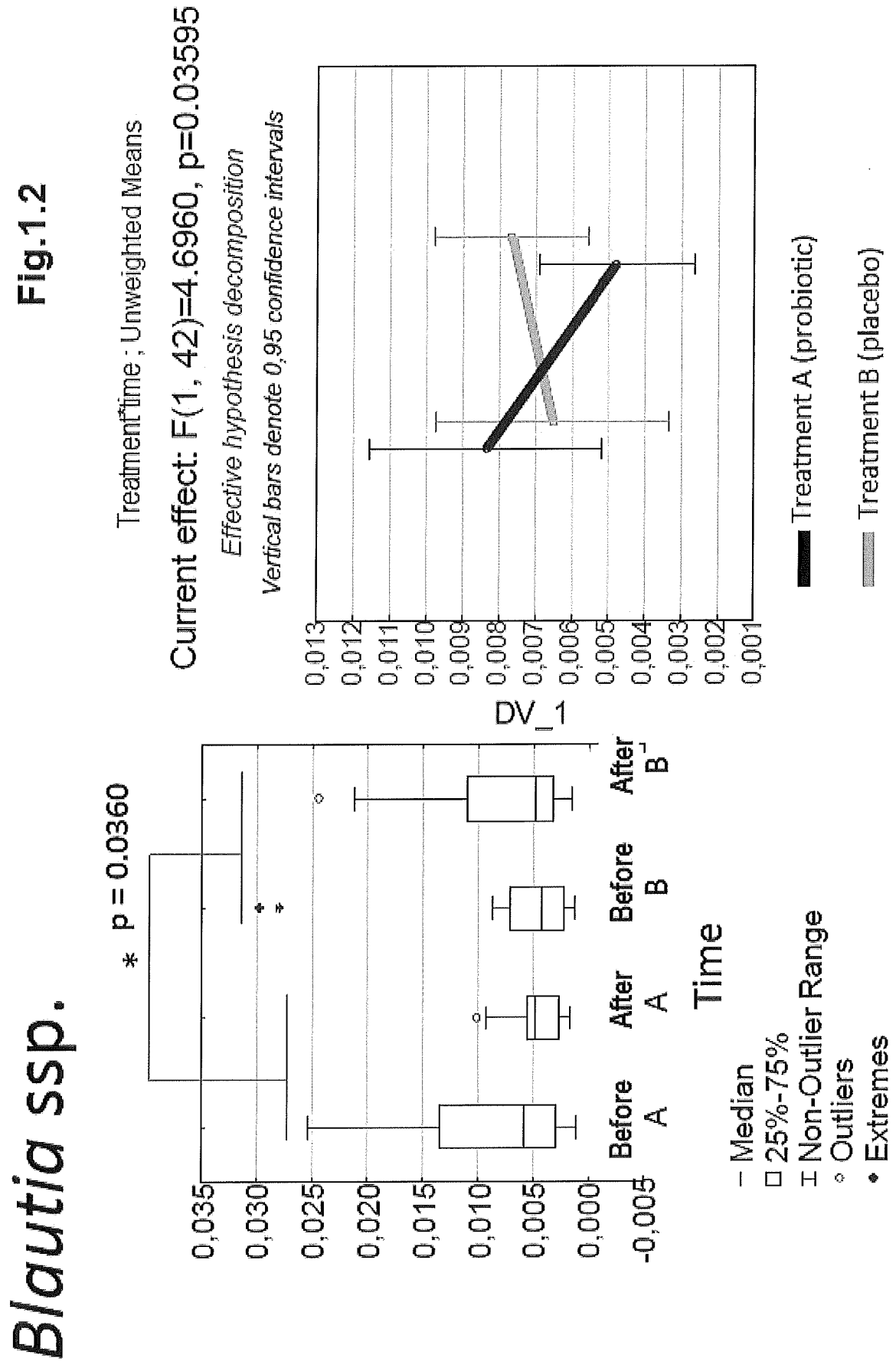


Fig.1.2


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
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
DV_1
Treatment A (probiotic)
Treatment B (placebo)

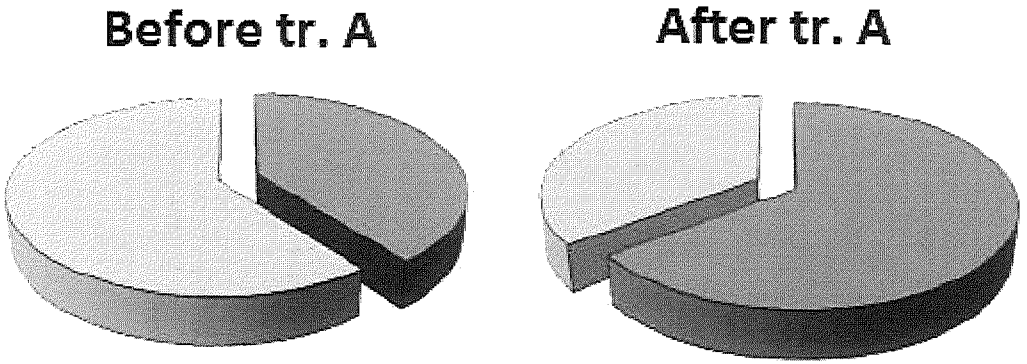

Before tr. A
After tr. A

Fig.2.1

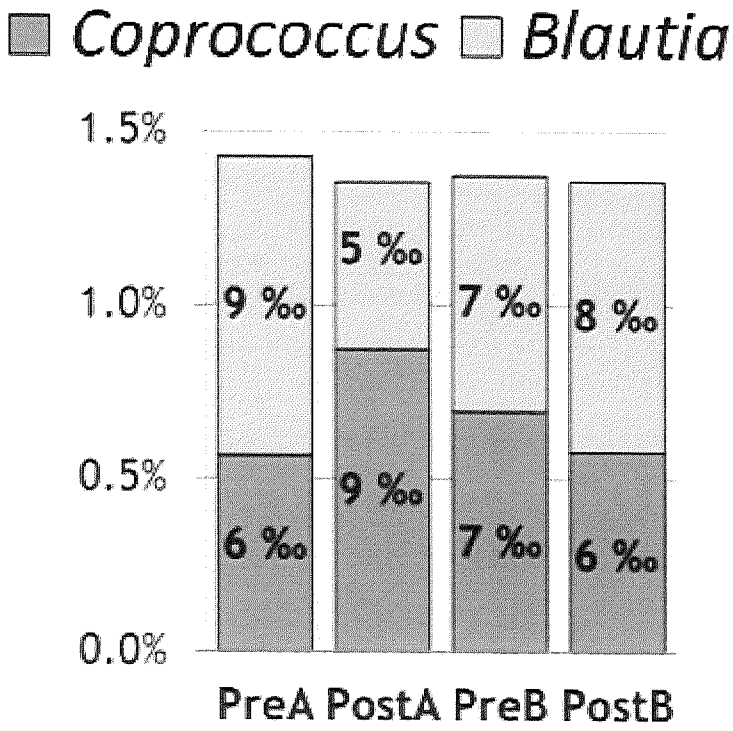

Coprococcus
Blautia
1.5%
1.0%
0.5%
0.0%
9 ‰
5 ‰
7 ‰
8 ‰
6 ‰
9 ‰
7 ‰
6 ‰
PreA PostA PreB PostB

Fig.2.2

Fig.3

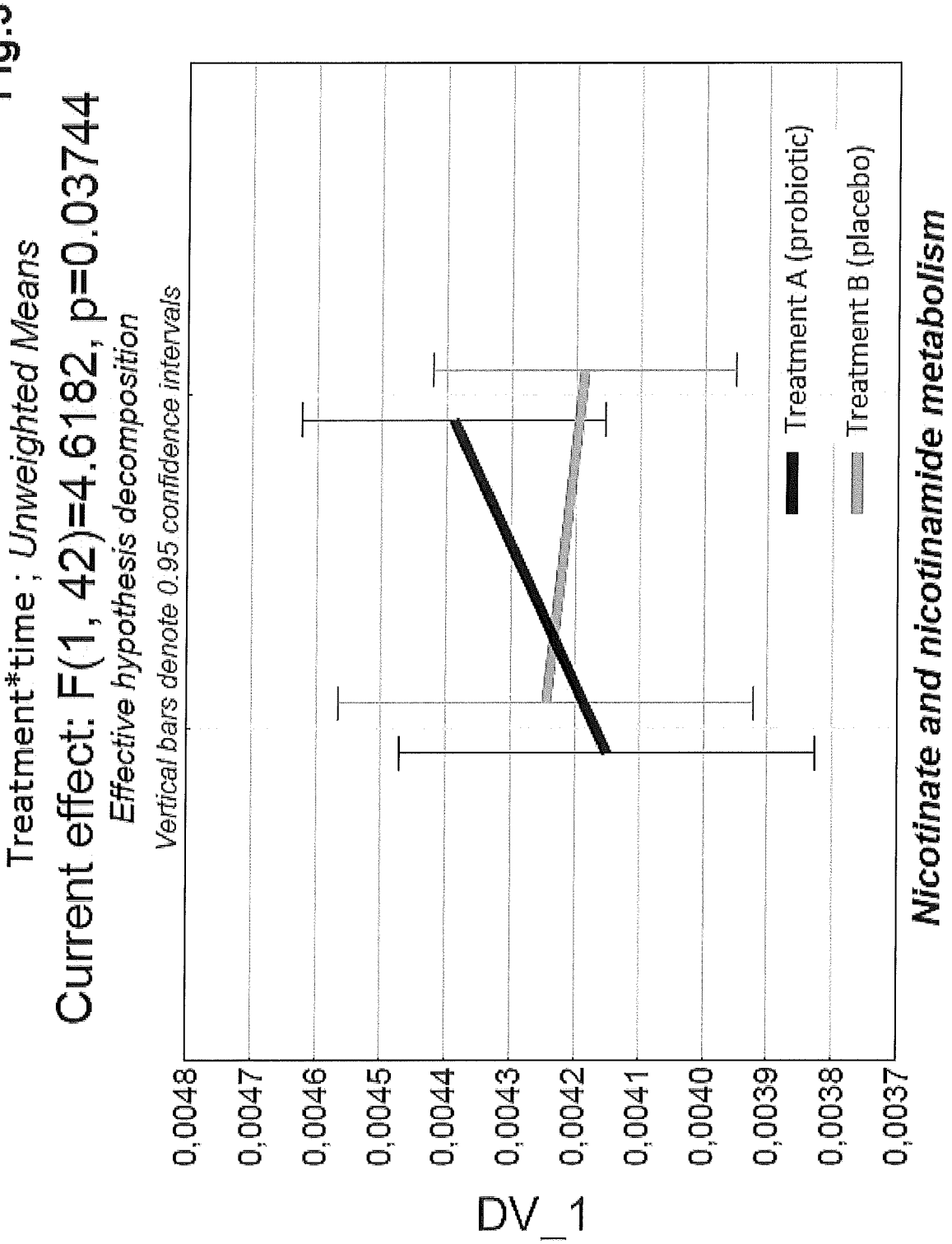
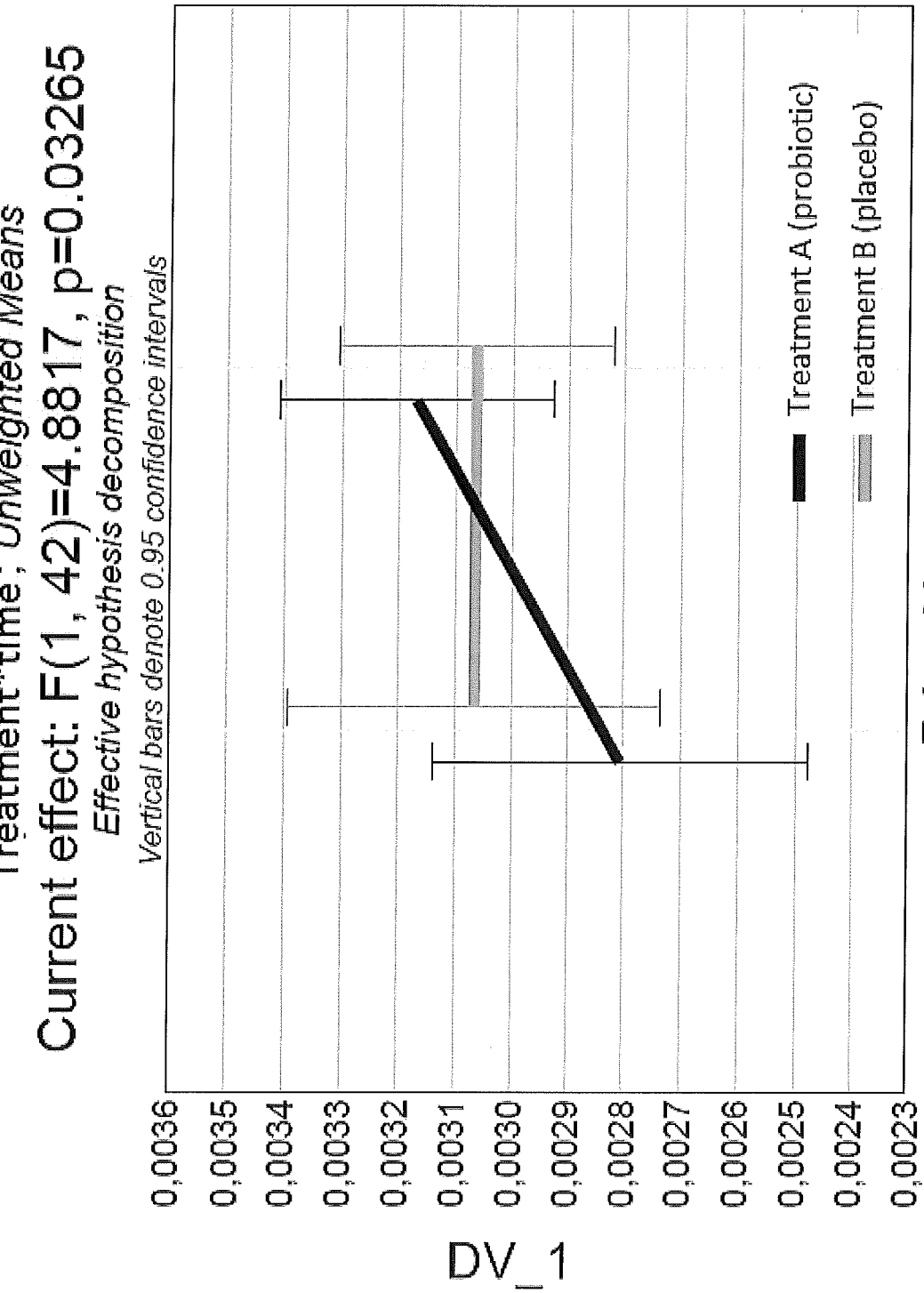

Treatment*time ; Unweighted Means
Current effect: F(1, 42)=4.6182, p=0.03744
Effective hypothesis decomposition
Vertical bars denote 0.95 confidence intervals
Nicotinate and nicotinamide metabolism
DV_1
0,0048
0,0047
0,0046
0,0045
0,0044
0,0043
0,0042
0,0041
0,0040
0,0039
0,0038
0,0037
Treatment A (probiotic)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

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
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
DV_1
Treatment A (probiotic)
Treatment B (placebo)
Folate biosynthesis

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/064284

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/10 A23L1/30 A61K35/74 C12Q1/68 C12R1/225
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)
C12Q A23L A61K C12R

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/022727 A1 (VRI BIOMEDICAL LTD [AU]; CONWAY PARICIA LYNNE [AU]) 18 March 2004 (2004-03-18) the whole document page 2, line 25 - page 6; figures 10-16 pages 11,20 pages 23-39; examples 5,6,10 page 19, line 23 - page 22; example 4 see page 20, line 30 to page 21, line 8 pages 16,19	1-16
Y	WO 2007/071815 A1 (CYFLO OY [FI]; KORKEAMAEKI MIKA [FI]; VAAHTOVUO JUSSI [FI]; MUNUKKA EV) 28 June 2007 (2007-06-28) the whole document page 2, line 35 - page 10, line 36; claims 1-21	1-16

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

"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	Date of mailing of the international search report
14 January 2015	26/01/2015

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/170617 A1 (FINEGOLD SYDNEY M [US]) 2 September 2004 (2004-09-02) the whole document paragraph [0007]; claims 1-17 paragraphs [0005] - [0012], [0050] - [0058]	1-16
Y	CN 1 840 206 A (UNIV SHANGHAI JIAOTONG [CN]) 4 October 2006 (2006-10-04) claims 1-10 the whole document	1-16
Y	WO 2005/001109 A2 (NAT INST HEALTH [US]; NYAN DOUGBEH C [US]; COLEMAN WILLIAM G JR [US];) 6 January 2005 (2005-01-06) the whole document pages 3-5; claims 19-35 pages 7,8,11 - page 16	1-16
Y	US 2010/112564 A1 (ZHAO LIPING [CN] ET AL) 6 May 2010 (2010-05-06) the whole document paragraphs [0008] - [0012], [0020] - [0026]; claims 1-11	1-16
Y	WO 2005/083122 A2 (STICHTING STREEKLABORATORIUM V [NL]; VAN ZWET ARIE ANTON [NL] ID VOOR) 9 September 2005 (2005-09-09) claims 5-10	1-16
Y	US 2009/061446 A1 (NIIMI HIDEKI [JP] ET AL) 5 March 2009 (2009-03-05) sequence 21 the whole document paragraphs [0013], [0014], [0021] - [0032], [0043] - [0056], [0117] - [0119]; claims 1-9	16
Y	WO 2006/050479 A2 (UNIV GEORGE MASON [US]; GILLEVET PATRICK [US]) 11 May 2006 (2006-05-11) the whole document claims 1-24	1-16

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PLANT ET AL: "Association of lactobacillus spp. with Peyer's Patches in Mice", CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 8, no. 2, 1 January 2001 (2001-01-01) , pages 320-324, XP002990497, ISSN: 1071-412X, DOI: 10.1128/CDLI.8.2.320-324.2001 the whole document abstract; figures 1-4; tables 1,2 -----	1-16
Y	F. SAVINO ET AL: "Lactobacillus reuteri DSM 17938 in Infantile Colic: A Randomized, Double-Blind, Placebo-Controlled Trial", PEDIATRICS, vol. 126, no. 3, 16 August 2010 (2010-08-16), pages e526-e533, XP055119050, ISSN: 0031-4005, DOI: 10.1542/peds.2010-0433 the whole document abstract -----	1-16
A	"Example Cross-Over Study Design (A Phase II, Randomized, Double-Blind Crossover Study of Hypertena and Placebo in Participants with High Blood Pressure) Methods Study Design", , October 2012 (2012-10), XP055119522, Retrieved from the Internet: URL:http://prsinfo.clinicaltrials.gov/trainTrainer/Crossover-Design-Fiction-Manuscript.pdf [retrieved on 2014-05-22] the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2014/064284

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004022727 A1	18-03-2004	AT 426014 T	15-04-2009
		BR 0314060 A	19-07-2005
		CA 2497989 A1	18-03-2004
		CN 1701116 A	23-11-2005
		DK 1539927 T3	13-07-2009
		EP 1539927 A1	15-06-2005
		ES 2324532 T3	10-08-2009
		JP 4455333 B2	21-04-2010
		JP 2005537791 A	15-12-2005
		KR 20050057259 A	16-06-2005
		NZ 538640 A	31-05-2007
		US 2006067921 A1	30-03-2006
		US 2010074878 A1	25-03-2010
		WO 2004022727 A1	18-03-2004
WO 2007071815 A1	28-06-2007	EP 1963520 A1	03-09-2008
		FI 20051319 A	23-06-2007
		US 2009053756 A1	26-02-2009
		WO 2007071815 A1	28-06-2007
US 2004170617 A1	02-09-2004	NONE	
CN 1840206 A	04-10-2006	NONE	
WO 2005001109 A2	06-01-2005	NONE	
US 2010112564 A1	06-05-2010	CN 101240315 A	13-08-2008
		US 2010112564 A1	06-05-2010
WO 2005083122 A2	09-09-2005	AU 2005217124 A1	09-09-2005
		CA 2557543 A1	09-09-2005
		EP 1730302 A2	13-12-2006
		US 2007065851 A1	22-03-2007
		WO 2005083122 A2	09-09-2005
US 2009061446 A1	05-03-2009	EP 1997886 A1	03-12-2008
		ES 2426038 T3	18-10-2013
		JP 4590573 B2	01-12-2010
		US 2009061446 A1	05-03-2009
		WO 2007097323 A1	30-08-2007
WO 2006050479 A2	11-05-2006	CA 2587670 A1	11-05-2006
		EP 1815016 A2	08-08-2007
		EP 2280085 A2	02-02-2011
		ES 2391744 T3	29-11-2012
		US 2009197249 A1	06-08-2009
		US 2014179537 A1	26-06-2014
		WO 2006050479 A2	11-05-2006

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 105518150 A
(43)申请公布日 2016.04.20

(21)申请号 201480049288.X

(22)申请日 2014.09.05

(30)优先权数据
MI2013A001473 2013.09.06 IT

(85)PCT国际申请进入国家阶段日
2016.03.07

(86)PCT国际申请的申请数据
PCT/IB2014/064284 2014.09.05

(87)PCT国际申请的公布数据
WO2015/033304 EN 2015.03.12

(71)申请人 索发股份公司
地址 意大利米兰

(72)发明人 A·比费 R·罗西 W·斐奥
S·D·古列尔梅蒂

(74)专利代理机构 上海专利商标事务所有限公司 31100
代理人 杨昀 陶家蓉

(51)Int.Cl.
C12Q 1/14(2006.01)
C12Q 1/04(2006.01)
C12Q 1/68(2006.01)
A23L 33/135(2016.01)
A61K 35/747(2015.01)
A61P 1/00(2006.01)
C12R 1/225(2006.01)

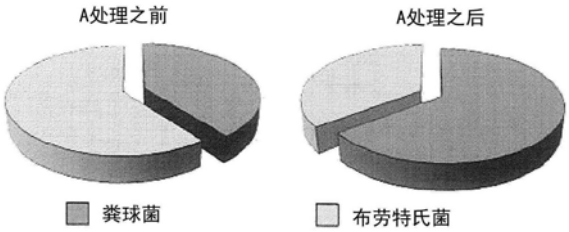
权利要求书2页 说明书10页
序列表1页 附图5页

(54)发明名称

用于评价包含微生物的组合物对肠道微生物群的作用的方法

(57)摘要

本发明涉及用于确定包含微生物（尤其是细菌）的组合物的益生/副益生活性的方法，所述方法基于，通过宏基因组学分析评价在摄取所述组合物之后，粪便微生物群中的定性和/或定量变化。此外，本发明涉及用于进行所述方法的试剂盒。



CN 105518150 A

1.一种用于确定个体粪便微生物群的组成变化的体外方法,所述变化为个体按照随机、双盲、安慰剂-对照的交叉方案摄入包含微生物的组合物和安慰剂之后的粪便微生物群的组成变化,所述方法包括如下步骤:

(i)在按照所述方案接受所述组合物或安慰剂之前和/或期间和/或之后,收集关于所述个体的健康状况和/或饮食习惯的信息;

(ii)在按照所述方案摄入所述组合物或安慰剂之前和/或期间和/或之后,从所述个体获取粪便样品;

(iii)通过对步骤(ii)中获取的粪便样品进行宏基因组学分析,来分析所述微生物群;

(iv)比较按照所述方案接受所述组合物或安慰剂之前和/或期间和/或之后的所述个体的粪便微生物群,所述比较优选定性地和/或定量地比较。

2.如权利要求1所述的方法,其特征在于,所述微生物是以单独或组合方式摄取的细菌和/或酵母。

3.如权利要求1或2所述的方法,其特征在于,所述微生物属于选自下组的属:乳杆菌(Lactobacillus)、双歧杆菌(Bifidobacterium)、芽孢杆菌(Bacillus)、丙酸杆菌(Propionibacterium)、链球菌(Streptococcus)、乳球菌(Lactococcus)、气球菌(Aerococcus)和肠球菌(Enterococcus);优选所述微生物是乳杆菌和/或双歧杆菌属的细菌。

4.如权利要求1-3中任一项所述的方法,其特征在于,所述微生物是乳杆菌属的细菌,其选自如下物种:副干酪乳杆菌(Lactobacillus paracasei)、嗜酸乳杆菌(Lactobacillus acidophilus)、解淀粉类乳杆菌(Lactobacillus amylolyticus)、嗜淀粉乳杆菌(Lactobacillus amylovorus)、食品乳杆菌(Lactobacillus alimentarius)、鸟乳杆菌(Lactobacillus aviaries)、短乳杆菌(Lactobacillus brevis)、布氏乳杆菌(Lactobacillus buchneri)、干酪乳杆菌(Lactobacillus casei)、纤维二糖乳杆菌(Lactobacillus cellobiosus)、棒状乳杆菌(Lactobacillus coryniformis)、卷曲乳杆菌(Lactobacillus crispatus)、弯曲乳杆菌(Lactobacillus curvatus)、德氏乳杆菌(Lactobacillus delbrueckii)、香肠乳杆菌(Lactobacillus farciminis)、发酵乳杆菌(Lactobacillus fermentum)、鸡乳杆菌(Lactobacillus gallinarum)、加氏乳杆菌(Lactobacillus gasseri)、瑞士乳杆菌(Lactobacillus helveticus)、希氏乳杆菌(Lactobacillus hilgardii)、约氏乳杆菌(Lactobacillus johnsonii)、马乳样乳杆菌(Lactobacillus kefiranofaciens)、开菲尔乳杆菌(Lactobacillus kefiri)、粘膜乳杆菌(Lactobacillus mucosae)、面包乳杆菌(Lactobacillus panis)、丘状乳杆菌(Lactobacillus collinoides)、类植物乳杆菌(Lactobacillus paraplantarum)、戊糖乳杆菌(Lactobacillus pentosus)、植物乳杆菌(Lactobacillus plantarum)、蓬蒂乳杆菌(Lactobacillus pontis)、罗伊氏乳杆菌(Lactobacillus reuteri)、鼠李糖乳杆菌(Lactobacillus rhamnosus)、清酒乳杆菌(Lactobacillus sakei)、唾液乳杆菌(Lactobacillus salivarius)和旧金山乳杆菌(Lactobacillus sanfranciscensis)。

5.如权利要求1-4中任一项所述的方法,其特征在于,所述微生物是副干酪乳杆菌物种的细菌,更优选其为菌株副干酪乳杆菌DG。

6.如权利要求1-5中任一项所述的方法,其特征在于,所述组合物中存在的所述微生物

的量是10至500亿个集落形成单位(CFU)的微生物,优选150至300,更优选200至250亿CFU的微生物。

7.如权利要求1-6中任一项所述的方法,其特征在于,所述组合物中存在的所述微生物是活的或死的微生物,或是裂解物或提取物的形式。

8.如权利要求1-7中任一项所述的方法,其特征在于,所述包含微生物的组合物被配制用于经口给予,优选以固体形式,优选以丸剂、胶囊剂、片剂、粒状粉末、硬胶囊、水溶性颗粒、囊剂或粒料。

9.如权利要求1-8中任一项所述的方法,其特征在于,所述宏基因组学分析包括如下步骤中的至少一个,优选全部:

-从所述粪便样品提取核酸,优选DNA;和

-对该粪便微生物群中存在的微生物进行分子分型。

10.如权利要求9所述的方法,其特征在于,对于粪便微生物群的分型通过分析核糖体的16S亚基的至少部分编码基因的核苷酸序列来进行。

11.如权利要求9或10所述的方法,其特征在于,对于粪便微生物群的分型通过采用PCR扩增核糖体的16S亚基的至少部分编码基因的核苷酸序列来进行。

12.如权利要求11所述的方法,其特征在于,所述PCR采用SEQ ID NO:1和2进行。

13.如权利要求11或12所述的方法,其特征在于,对扩增的核苷酸序列测序,优选采用离子激流测序技术。

14.如权利要求9-13中任一项所述的方法,其特征在于,通过分级聚类程序和/或分类学分析,和/或通过构建系统树,优选采用热图,来表征所述微生物。

15.如权利要求14所述的方法,其特征在于,通过采用参数和/或非参数统计学方法来分析所述表征的结果。

16.一种用于进行权利要求1-15中任一项所述的方法的试剂盒,所述试剂盒包含:

-所述试剂盒的标识码;

-包含微生物的组合物的至少一种口服制剂,所述微生物优选属于副干酪乳杆菌物种,更优选菌株副干酪乳杆菌DG,所包含微生物的量是10至500亿个集落形成单位(CFU)的微生物,优选150至300,更优选200至250亿CFU的微生物;

-不包含微生物的安慰剂的至少一种口服制剂;

所述微生物的组合物按照以所述安慰剂为对照的随机、双盲交叉方案摄取,并且,包含所述微生物的所述组合物和安慰剂通过编码来鉴定。

用于评价包含微生物的组合物对肠道微生物群的作用的方法

[0001] 本发明涉及用于确定包含微生物(尤其是细菌)的组合物的益生/副益生活性的方法,所述方法基于评价在摄取所述组合物之后,粪便微生物群中的定性和/或定量变化。此外,本发明涉及用于进行所述方法的试剂盒。

[0002] 胃肠道包含多种微生物群体,其在每个个体的发育过程中发展并增殖,形成所谓肠道微生物群或肠道菌群。

[0003] 因此,肠道微生物群是一个高度复杂的生态系统,其由不同微生物群体之间的平衡调节而成,或所谓生态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粘膜的发育和内稳态。

[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),会导致丧失肠道内稳态,由此会决定广谱病理学的发病机理。

[0005] 为了处理肠道失调状况,或者在任何情况下,为了维持肠道微生物群的平衡,摄取益生/副益生产品的实践愈发地普遍。

[0006] 按照FAO/WHO的定义,益生剂是一组"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"。

[0007] 根据上述定义,与方法的开发相关的益处使其能够快速且可靠地评价包含微生物的外生组合物/制剂对个体的肠道微生物群的细菌组成的相当明显的影响。

[0008] 事实上,基于采用该方法检测的作用,即基于摄入包含微生物的组合物将如何定量地和/或定性地改变肠道微生物群,能够建立所述组合物是否能够有利于和/或确保人体健康,以及因此,其是否能够满足被鉴定为益生/副益生剂的基础资格条件之一。

[0009] 本发明通过如下方式来满足上述要求:提供通过分子分析来确定在按照随机、双盲、安慰剂-对照交叉方案摄入了包含微生物(优选细菌)的组合物之后,个体的粪便微生物群的组成的定性和/或定量变化的方法。

[0010] 事实上,申请人已通过实验第一次证明,进行,尤其是对健康群体进行交叉干预研究方案,以防止显著个体间变异性会隐藏处理(具体而言,采用益生/副益生剂的粗粒)的可能作用,或防止显著的个体间变异性导致统计学假阳性。

[0011] 本发明方法,除了特别有利于确定包含微生物(即假定的益生/副益生剂)的一般组合物对粪便微生物群的作用之外,还有利于确定已知益生/副益生剂对人体的促健康作用,或用于确定已知益生/副益生剂的任何新的、特定的作用,例如,通过研究在摄取所述组合物之后刺激和/或抑制了哪些微生物群体的生长。事实上,基于在摄取所述组合物之后,所涉及的其生长被刺激和/或被抑制的微生物群体的主要活性,能够确定所述组合物的可能的新作用。例如,如果,在摄取了本发明方法的益生剂之后,发现特定的细菌群以定量方式生长,并且该细菌群的代谢主要涉及例如丁酸的生成,则可推定,能够通过接受所述益生剂来增加肠道中丁酸的量。

[0012] 本发明方法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。

[0013] 为了能够更好地理解详细说明，本发明附有图1-4：

[0014] -图1显示统计学分析的结果，其评价用本发明组合物处理之前和之后(A)粪球菌(Coprococcus)属的细菌群的增加(图1.1)和布劳特氏菌(Blautia)属细菌群的减少(图1.2)，以及同时用安慰剂处理之前和之后(B)粪球菌属的细菌群的减少(图1.1)和布劳特氏菌属的细菌群的增加(图1.2)；

[0015] -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)；

[0016] -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)；

[0017] -图3显示统计学分析的结果，其用于建立，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；和

[0018] -图4显示统计学分析的结果，其用于建立，在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。

[0019] 本发明的第一方面涉及确定粪便微生物群的组成变化的方法，所述粪便微生物群为个体按照随机、双盲、安慰剂-对照的交叉方案摄入包含微生物的组合物/制剂之后的的粪便微生物群，所述方法包括如下步骤：

[0020] a)在按照随机、双盲、安慰剂-对照的交叉方案接受所述组合物或安慰剂之前和/或期间和/或之后，收集关于所述个体的健康状况和/或饮食习惯的信息；

[0021] b)获得在按照随机、双盲、安慰剂-对照的交叉方案摄入所述组合物或安慰剂之前和/或期间和/或之后，来自所述个体的至少一个粪便样品；

[0022] c)通过对步骤b)的粪便样品进行的宏基因组学分析来分析所述微生物群；

[0023] d)比较按照随机、双盲、安慰剂-对照的交叉方案摄入所述组合物或安慰剂之前和/或期间和/或之后的所述个体的粪便微生物群，优选定性地和/或定量地比较。

[0024] 在本发明内容中，术语粪便微生物群指的是存在于个体粪便中的整个微生物群体，并且其反映所述个体的肠中存在的整个微生物群体。因此，术语粪便微生物群在本文中指的是肠道微生物群的同义词。具体而言，包括在本发明组合物中的微生物是单独或组合形式的细菌和/或酵母和/或其它微生物。

[0025] 包含细菌的组合物特别优选用于本发明目的。具体而言，所述细菌属于选自下组的属：乳杆菌(Lactobacillus)、双歧杆菌(Bifidobacterium)、芽孢杆菌(Bacillus)、丙酸杆菌(Propionibacterium)、链球菌(Streptococcus)、乳球菌(Lactococcus)、气球菌(Aerococcus)和肠球菌(Enterococcus)。更优选地，所述细菌属于乳杆菌和/或双歧杆菌属。

[0026] 具体而言，所述乳杆菌选自：副干酪乳杆菌(Lactobacillus paracasei)、嗜酸乳杆菌(Lactobacillus acidophilus)、解淀粉类乳杆菌(Lactobacillus amylolyticus)、嗜淀粉乳杆菌(Lactobacillus amylovorus)、食品乳杆菌(Lactobacillus alimentarius)、鸟乳杆菌(Lactobacillus aviaries)、短乳杆菌(Lactobacillus brevis)、布氏乳杆菌(Lactobacillus buchneri)、干酪乳杆菌(Lactobacillus casei)、纤维二糖乳杆菌

(Lactobacillus cellobiosus)、棒状乳杆菌(Lactobacillus coryniformis)、卷曲乳杆菌(Lactobacillus crispatus)、弯曲乳杆菌(Lactobacillus curvatus)、德氏乳杆菌(Lactobacillus delbrueckii)、香肠乳杆菌(Lactobacillus farciminis)、发酵乳杆菌(Lactobacillus fermentum)、鸡乳杆菌(Lactobacillus gallinarum)、加氏乳杆菌(Lactobacillus gasseri)、瑞士乳杆菌(Lactobacillus helveticus)、希氏乳杆菌(Lactobacillus hilgardii)、约氏乳杆菌(Lactobacillus johnsonii)、马乳样乳杆菌(Lactobacillus kefiranofaciens)、开菲尔乳杆菌(Lactobacillus kefiri)、粘膜乳杆菌(Lactobacillus mucosae)、面包乳杆菌(Lactobacillus panis)、丘状乳杆菌(Lactobacillus collinoides)、类植物乳杆菌(Lactobacillus paraplantarum)、戊糖乳杆菌(Lactobacillus pentosus)、植物乳杆菌(Lactobacillus plantarum)、蓬蒂乳杆菌(Lactobacillus pontis)、罗伊氏乳杆菌(Lactobacillus reuteri)、鼠李糖乳杆菌(Lactobacillus rhamnosus)、清酒乳杆菌(Lactobacillus sakei)、唾液乳杆菌(Lactobacillus salivarius)和旧金山乳杆菌(Lactobacillus sanfranciscensis)。

[0027] 对于本发明的目的特别优选的是属于副干酪乳杆菌物种的细菌,更优选副干酪乳杆菌DG菌株。

[0028] 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。所述菌株的最初命名是干酪乳杆菌DG干酪菌种(Lactobacillus casei DG sub.casei)。

[0029] 具体而言,双歧杆菌属的细菌选自:青春双岐杆菌(Bifidobacterium adolescentis)、动物双歧杆菌(Bifidobacterium animalis)、两岐双岐杆菌(Bifidobacterium bifidum)、短双岐杆菌(Bifidobacterium breve)和长双歧杆菌(Bifidobacterium longum)。

[0030] 所述酵母优选属于酵母属(Saccharomyces),更优选属于酿酒酵母(Saccharomyces cerevisiae)物种。

[0031] 一般而言,本发明组合物中包含的微生物是EFSA的QPS列表中指定的个体微生物或任何微生物物种的组合

[0032] (http://www.efsa.europa.eu/it/search/doc/3020.pdf).

[0033] 本发明组合物的微生物优选是活的,由此所述组合物也可被定义为益生剂。

[0034] 或者,所述组合物的微生物是死的,和/或是裂解物或提取物的形式,由此所述组合物也可被定义为副益生剂。因此,本发明组合物还可以是已知的或假定的益生剂或副益生剂。

[0035] 在本发明的一个实施方式中,所述组合物包含约10亿至500亿个集落形成单位(CFU)的微生物,优选150至300亿,更优选200至250亿个CFU的微生物。

[0036] 在本发明的一个实施方式中,所述组合物配制用于经口给予。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。

[0037] 或者,本发明的组合物被配制为液体,例如糖浆或饮品,或者被添加至食物,例如酸奶、乳酪,或果汁。

[0038] 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。

[0039] 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剂

和/或药物产品的赋形剂。

[0040] 在本发明的另一个实施方式中，本发明的组合物富含维生素、微量元素例如锌和硒，酶和/或益生元物质，例如，低聚果糖(FOS)、低聚半乳糖(GOS)、菊粉，瓜尔豆胶或其组合。

[0041] 就该组合物的摄取而言，如先前所解释的，其按照随机、双盲、安慰剂-对照的交叉方案摄取。换言之，在摄取过程中，试验中涉及的研究者和个体均不知晓被分配何种处理(所述处理是不可分辨的、双盲的)，并且，相同的个体按照随机顺序在不同时间接触包含微生物的组合物和安慰剂的处理(交叉)。

[0042] 在本发明的一个实施方式中，所述方案包括如下阶段：1)预招募期，其中所述个体优选不摄取包含微生物的组合物或安慰剂；和/或2)第一处理期，其中所述个体优选摄取包含微生物的组合物或安慰剂；和/或3)清洗期(wash-out phase)，其中所述个体优选不摄取包含微生物的组合物或安慰剂；和/或4)第二处理期，其中所述个体优选摄取安慰剂或包含微生物的组合物。

[0043] 如上所述，第2阶段和第4阶段的摄取以随机、双盲的方式进行。明确的是，在第1阶段摄取安慰剂的个体将在第2阶段摄取包含微生物的组合物，反之亦然。

[0044] 该方案的不同阶段的时间长度优选是相同的。具体而言，这些阶段中至少一个(优选全部阶段)的时间长度是约四周。

[0045] 在本发明内容中，术语清洗指的是在摄取包含微生物的组合物或安慰剂的两个阶段之间的过程，其中所述个体不摄取任何试剂，并因此应"排出"他或她先前已摄取的物质，即不包括任何处理以清除所有残余效应的过程。

[0046] 在本发明的一个实施方式中，本发明组合物优选一天摄取一次，更优选在睡醒后的即刻。

[0047] 或者，夜间摄取也是可行的，优选餐后至少3小时。

[0048] 收集关于所述个体的健康状况和/或饮食习惯的信息的步骤优选通过问卷形式收集所述信息的方式进行。所述问卷经特别定制以收集关于进行本发明方法的个体的健康状况和/或饮食习惯的数据。

[0049] 具体而言，所述问卷是标准版，其上列出关于所述个体的健康状况和/或饮食习惯的征询。关于健康状况，所述个体可采用与各问题相关联的打分标准来回答。打分标准优选是口述数字评分法(VNS)，或视觉模拟评分法(VAS)或口述描绘评分法(VRS)。关于饮食习惯，所述个体可通过指示他或她日常消耗的食物并且(若可能)确定消耗量来回答。

[0050] 收集信息的步骤优选在预招募期起始时和/或在第一处理期之前和/或之后和/或在第二处理期终末之前和/或之后进行。

[0051] 至少一个粪便样品的获取优选在第一处理器的起始和/或终末和/或第二处理期的起始和/或终末进行。

[0052] 所述粪便样品优选在不早于处理前48小时，更优选不早于处理前24小时的时间获取，或贮存在优选+4℃至-20℃的温度，更优选-20℃，贮存时长优选不超过7-10天。粪便样品在处理之前的贮存或在较低温度的贮存优选在室温进行。

[0053] 通过对粪便样品进行宏基因组学分析的微生物群分析步骤对于核酸进行，优选对于提取自粪便微生物群的DNA进行。

[0054] 具体而言，通过宏基因组学分析对微生物群进行的分析包括如下步骤中的至少一个，且优选全部：

[0055] • 从所述粪便样品提取核酸，优选DNA；和

[0056] • 对所述粪便微生物群进行分子分型。

[0057] 一般而言，从粪便样品提取核酸(具体是DNA)通过采用本领域技术人员已知用于该目的的方式进行。

[0058] 在本发明的一个实施方式中，对微生物群的分型通过分析编码核糖体的亚基，优选核糖体的16S亚基的基因(即编码16S rRNA分子的基因)的至少一部分进行核苷酸序列分析来进行。

[0059] 为此，从粪便样品提取的DNA采用本领域已知的技术(例如PCR)来扩增。优选地，所述扩增通过采用一对寡核苷酸(引物)来进行；

[0060] 优选通过采用SEQ ID NO:1(Probio_Uni 5'-CCTACGGGRSGCAGCAG-3')和SEQ ID NO:2(Probio_Rev 5'-ATTACCGCGGCTGCT-3')(Milani C,Hevia A,Foroni E,Duranti S,Turroni F等.(2013)"粪便微生物群评估：一种优化的离子激流16S rRNA基因分析方案(Assessing the Fecal Microbiota:An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol)".PLoS ONE 8(7):e68739)。

[0061] 进行PCR的条件可视需要扩增的核酸的质量和数量和/或所用的引物而变化。在任何情况中，对PCR条件的设定是本领域技术人员的常规技能。

[0062] 优选地，随后对扩增的核酸的部分进行测序。

[0063] 本领域技术人员可采用用于该目的的任何已知方法。优选地，所用方法选自：基于桑格测序法、焦磷酸测序法和离子激流测序法的测序。

[0064] 在离子激流的情况中，优选采用优选在5'端具有衔接子序列的引物。在本发明的特别优选的实施方式中，所述衔接子序列是SEQ ID NO:1和2。

[0065] 一旦获得所述序列，以及进而地，一旦所述粪便微生物群的微生物群体已被分型，即表征微生物群落，优选通过分级聚类程序或分类学分析和/或通过构建系统树，优选采用热图。就此而言，QIIME软件特别优选用于本发明目的。

[0066] 最后，获自表征分析的数据优选采用参数和/或非参数类型的统计学方法分析。

[0067] 本发明的另一个方面涉及用于进行本发明方法的试剂盒，所述试剂盒包含：

[0068] -该试剂盒的标识码；

[0069] -包含微生物的组合物的至少一种口服制剂，所述微生物优选属于副干酪乳杆菌物种，更优选菌株副干酪乳杆菌DG，包含微生物的量是10至500亿个集落形成单位(CFU)的微生物，优选150至300，更优选200至250亿CFU的微生物。

[0070] -不包含微生物的安慰剂的至少一种口服制剂；所述微生物的组合物按照以所述安慰剂为对照的随机、双盲、交叉方案摄取，并且，包含微生物和所述安慰剂的所述组合物通过编码来鉴定。

[0071] 所述安慰剂优选与所述包含微生物的组合物的口服制剂在外观上(即形式)相同，但与所述包含微生物的组合物的口服制剂具有本质差异，所述微生物优选属于副干酪乳杆菌物种，更优选菌株副干酪乳杆菌DG。安慰剂的口服制剂不包含微生物。

[0072] 在本发明的一个优选实施方式中，所述试剂盒包括含有含微生物的组合物的口服

制剂的至少28粒胶囊或片剂或丸剂或口腔片或硬胶囊或囊剂，并且，优选地，含有含安慰剂口服制剂的相等数量的片剂或丸剂或口腔片或硬胶囊或囊剂。

[0073] 根据本发明的一个优选实施方式，所述至少一种口服制剂是至少一种胶囊、至少一种片剂、至少一种丸剂、至少一种口腔片、至少一种硬胶囊、至少一种囊剂或至少一种颗粒剂。

[0074] 所述口服制剂通过编码鉴定，例如颜色编码、数字编码、字母编码，或字母数字混合编码。为实现所述方法的目的，所述编码将用于知晓何时摄入了包含微生物的组合物，以及何时摄入了安慰剂，如前所述。

[0075] 所述口服制剂是外观上(即，形式)相同，但本质不同的，因为一种含有包含微生物的组合物，且另一种含有安慰剂。此外，所述两种制剂各自由编码鉴定。

[0076] 以此方式，包含在试剂盒内的含有微生物的组合物和安慰剂是，例如，无法由任何个体区分的。此外，包含在试剂盒中的含有微生物的组合物和安慰剂能被任何编码(无论何种形式)鉴定，例如，颜色编码、数字编码、字母编码，或字母数字混合编码。

[0077] 该编码与物质性质的对应性，即其为包含微生物的组合物还是安慰剂，仅仅是该试剂盒的生产商所已知的。

[0078] 根据本发明的另一个实施方式，所述试剂盒还包含特别定制的问卷，以收集关于履行本发明方法的个体的健康状况的数据。

[0079] 具体而言，所述问卷是标准版，其上列出关于所述个体的健康状态和/或饮食习惯的问题。关于健康状态，所述个体可采用与各问题相关联的打分标准来回答。打分标准优选是口述数字评分法(VNS)，或视觉模拟评分法(VAS)或口述描绘评分法(VRS)。关于饮食习惯，所述个体可通过指明他或她日常消耗的食物并且(若可能)确定消耗量来回答。

[0080] 本发明的另一个方面，涉及所述试剂盒用于诊断和/或治疗目的的应用。

实施例

[0081] 处理

[0082] 对健康个体进行随机、双盲、安慰剂-对照的交叉的饮食干预研究。

[0083] 志愿者按照如下标准征募：

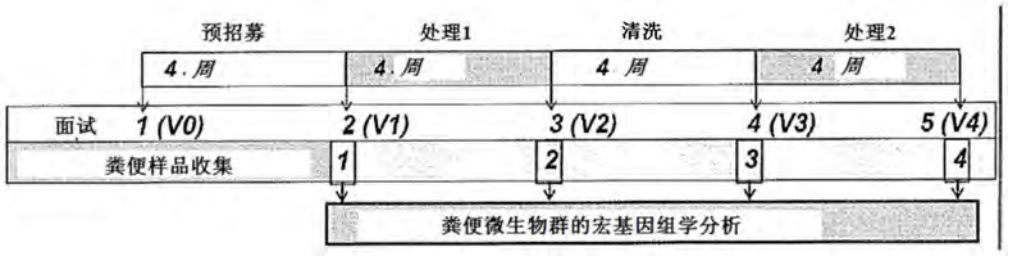
[0084] -入选标准：健康男性和女性，年龄在18和55岁之间；签署知情同意书；

[0085] -排除标准：在该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。

[0086] 该益生饮食干预根据交叉设计进行，如下表1所示。

[0087] 表I

[0088]



[0089] 在预登记(pre-enrolment)步骤(4周),志愿者遵循其常规饮食,不消耗益生发酵的乳品(因此,传统酸奶是允许的)、益生饮食补充剂,或益生元饮食补充剂。

[0090] 在预登记阶段末期,每天随机给予志愿者益生剂或安慰剂的一种胶囊,持续4周。

[0091] 举例而言,采用Enterolactis Plus作为待给予的益生剂;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。

[0092] 包含安慰剂的胶囊在外观上与包含益生剂的那些相同,其明确地缺乏益生剂。

[0093] 所述活性物质(即益生剂)的味道和颜色与安慰剂相同。

[0094] 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。

[0095] 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。

[0096] 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。

[0097] 总之,该研究涉及4个阶段,其各自持续4周:

[0098] • 预招募阶段:所述个体既不经历Enterolactis Plus处理,也不经历安慰剂处理。

[0099] • 处理1:所述个体经历Enterolactis Plus处理或安慰剂处理。

[0100] • 清洗:所述个体既不经历Enterolactis Plus处理,也不经历安慰剂处理。

[0101] 处理2:所述个体分别经历安慰剂处理或Enterolactis Plus处理。

[0102] 检测和样品收集。

[0103] 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次会面。

[0104] 在第一次会面期间,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)前来进行第二次会面,所述粪便样品在先前24小时时程之内收集于第一次会面交于的特定容器中。

[0105] 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。

[0106] 此外,在第二次会面过程中,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。

[0107] 并且,指示志愿者如何摄取所述产品。

[0108] 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)前来进行第三次会面,所述粪便样品在之前24小时之内收集。

[0109] 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。

[0110] 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。

[0111] 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。

[0112] 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)。

[0113] 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。

[0114] 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。

[0115] 粪便微生物群的分析

[0116] 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,采用宏基因组学策略;简言之,其由如下步骤组成:

[0117] 1.从粪便样品提取宏基因组学DNA,并对其定量和标准化;

[0118] 2.通过PCR扩增编码16S rRNA的细菌基因的V3高变区;

[0119] 3.对PCR产物定量;

[0120] 4.对扩增的产物测序;

[0121] 5.对序列进行生物信息学分析。

[0122] 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)。

[0123] 具体而言,SEQ ID NO:1和2引物对扩增16S rRNA基因的V3区域。

[0124] 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。

[0125] 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。

[0126] 进行PCR的条件如下:

[0127] • 95℃,5分钟;

[0128] • 94℃,30秒;55℃,30秒;和72℃,90秒;进行35个循环;

[0129] • 72℃,10分钟。

[0130] 在PCR结束时,通过电泳来检验扩增物的完整性。

[0131] 该方法的步骤5,是表征所述微生物所必需的。

[0132] 群落可采用目前已知用于该目的的多种技术进行。更具体地,利用:分级聚类、分类学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。

[0133] 数据的统计学分析

[0134] 所述统计学分析采用STATISTICA软件(史丹索特公司(Statsoft Inc.),美国俄克拉荷马州塔尔萨)进行。

[0135] 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。

[0136] 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。

[0137] 处理的结果

[0138] 该研究由总计22位个体(11位女性和11位男性)完成。

[0139] 初始时登记了33位个体,但其中11位因如下种种原因于早期退出: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剂(3),饮食习惯大幅变化(1),和,患上伴生腹泻的季节性流感(1)。

[0140] 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。

[0141] 当分析获自该分析的数据时,就分类而言,观察到该研究参与者的肠道微生物群的高稳定性。

[0142] 事实上,发现:

[0143] 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;

[0144] b)131种鉴定的家族中的11种占超过90%的序列;和

[0145] c)262种鉴定的属中的20个属占超过90%的序列。

[0146] 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。

[0147] 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隐藏益生剂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)的组中出现了关于属的统计学显著差异。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。

[0148] 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)。

[0149] 粪球菌是肠道水平的主要丁酸盐生成者之一。

[0150] 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)的饮食的处理的佐剂。

[0151] 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。

[0152] 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。

[0153] 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。

[0154] 因此,在采用益生剂处理之后,在本文中为给予副干酪乳杆菌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。

[0155] 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,因为在用益生剂处理之后(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。

[0156] 因此,在用益生剂处理之后,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。

[0157] 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剂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。

序列表

<110> 索发股份公司(Sofar SpA)

<120> 用于评价包含微生物的组合物对肠道微生物群的作用的方法

<130> 71.S0662.12.IT.1

<160> 2

<170> PatentIn version 3.3

<210> 1
<211> 17
<212> DNA
<213> 人工

[0001]

<220>
<223> 益生_正向

<400> 1
cctacgggrs gcagcag 17

<210> 2
<211> 15
<212> DNA
<213> 人工

<220>
<223> 益生-反向

<400> 2
attaccgcgg ctgct 15

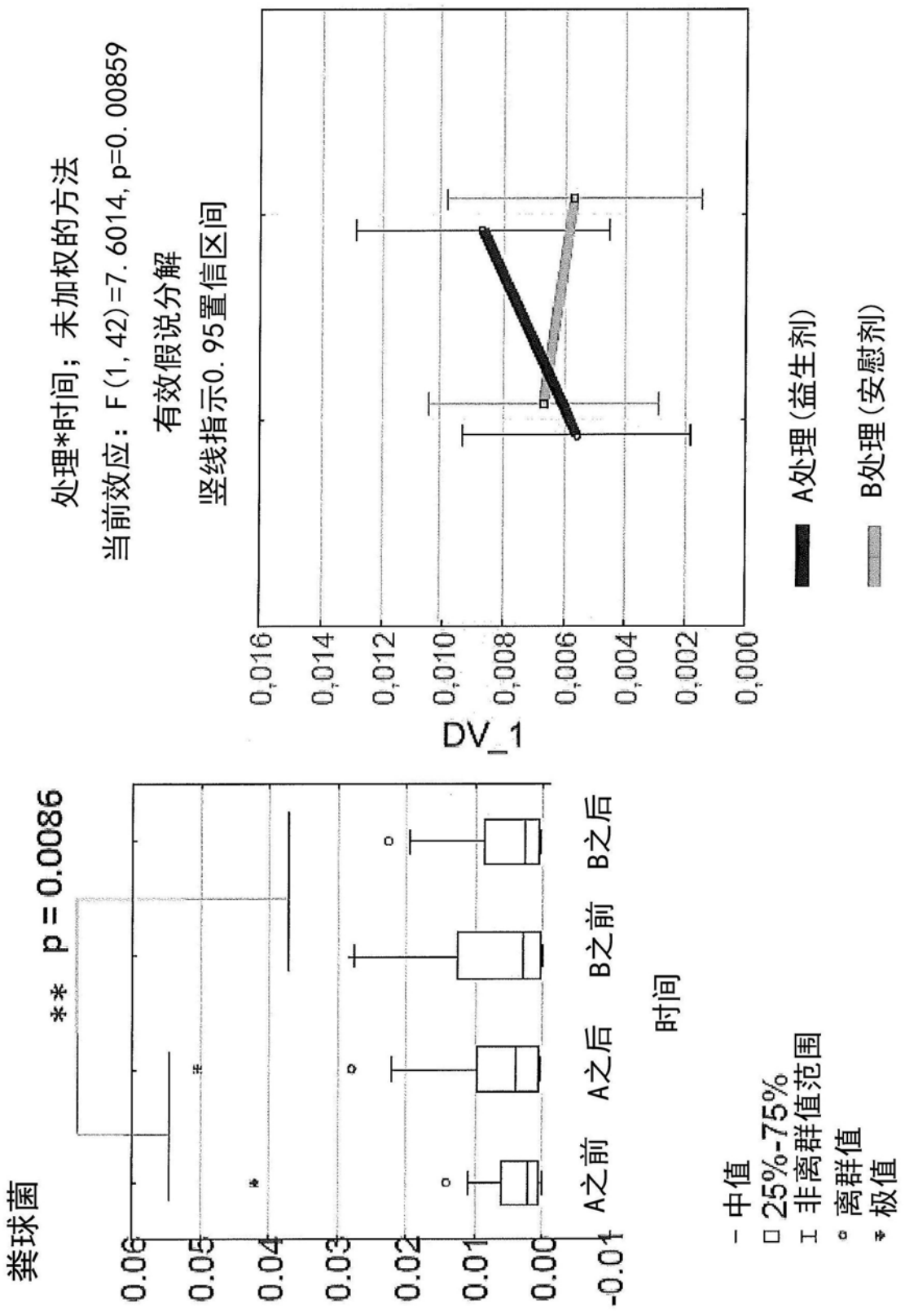

处理*时间；未加权的方法
当前效应：F(1, 42)=7.6014, p=0.00859
有效假说分解
竖线指示0.95置信区间
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
DV_1
A处理(益生剂)
B处理(安慰剂)
粪球菌
** p = 0.0086
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
A之前
A之后
B之前
B之后
时间
中值
25%-75%
非离群值范围
离群值
极值

图1.1

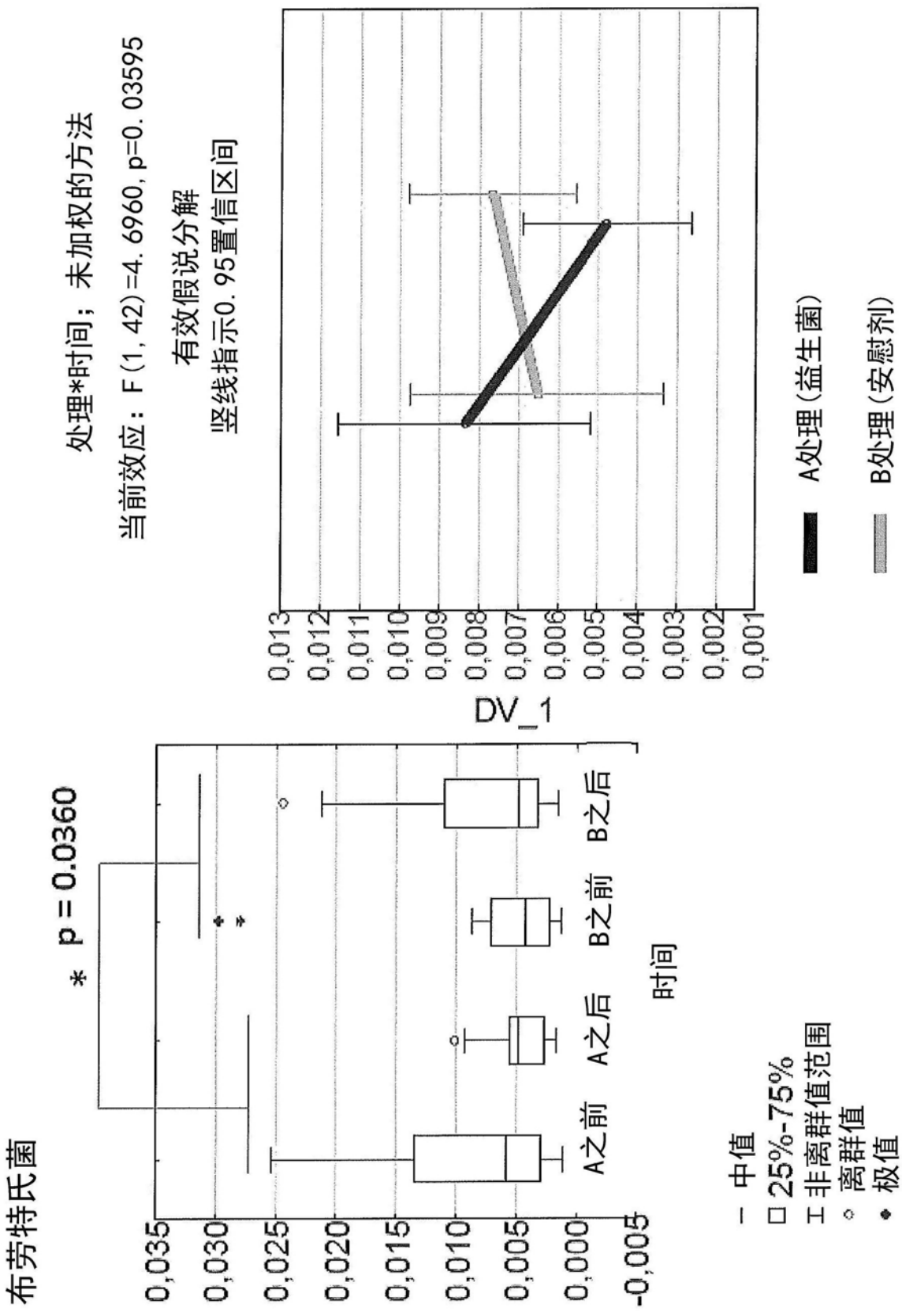

处理*时间；未加权的方法
当前效应：F(1, 42)=4. 6960, p=0. 03595
有效假说分解
竖线指示0. 95置信区间
A处理(益生菌)
B处理(安慰剂)
DV_1
0,013
0,012
0,011
0,010
0,009
0,008
0,007
0,006
0,005
0,004
0,003
0,002
0,001
布劳特氏菌
* p = 0.0360
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
-0,005
A之前
A之后
B之前
B之后
时间
中值
25%-75%
非离群值范围
离群值
极值

图1.2

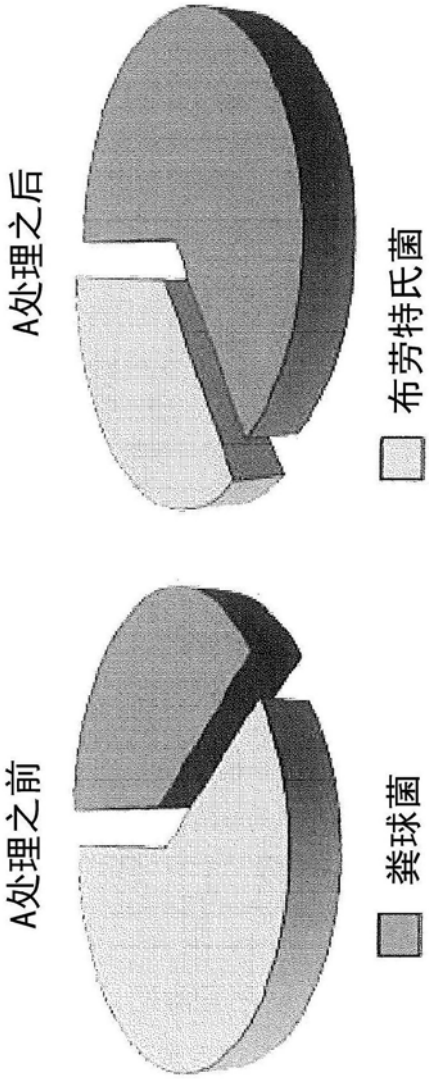


图2.1

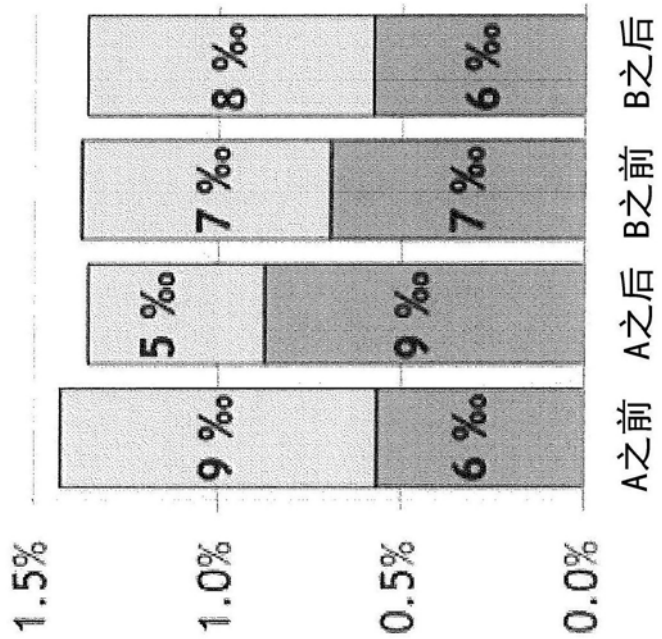


图2.2

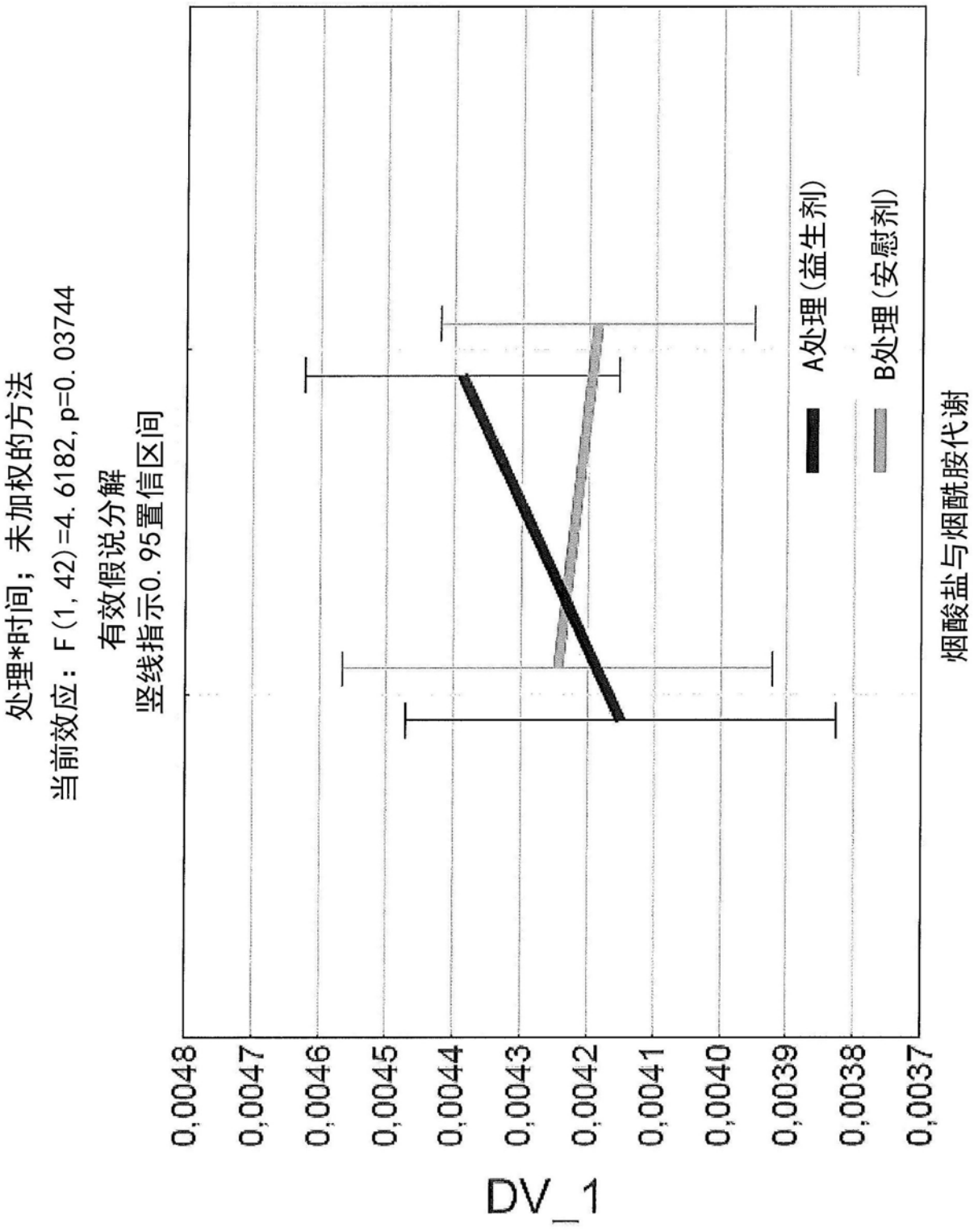

处理*时间；未加权的方法
当前效应：F(1, 42)=4. 6182, p=0. 03744
有效假说分解
竖线指示0. 95置信区间
0,0048
0,0047
0,0046
0,0045
0,0044
0,0043
0,0042
0,0041
0,0040
0,0039
0,0038
0,0037
DV_1
A处理(益生剂)
B处理(安慰剂)
烟酸盐与烟酰胺代谢

图3

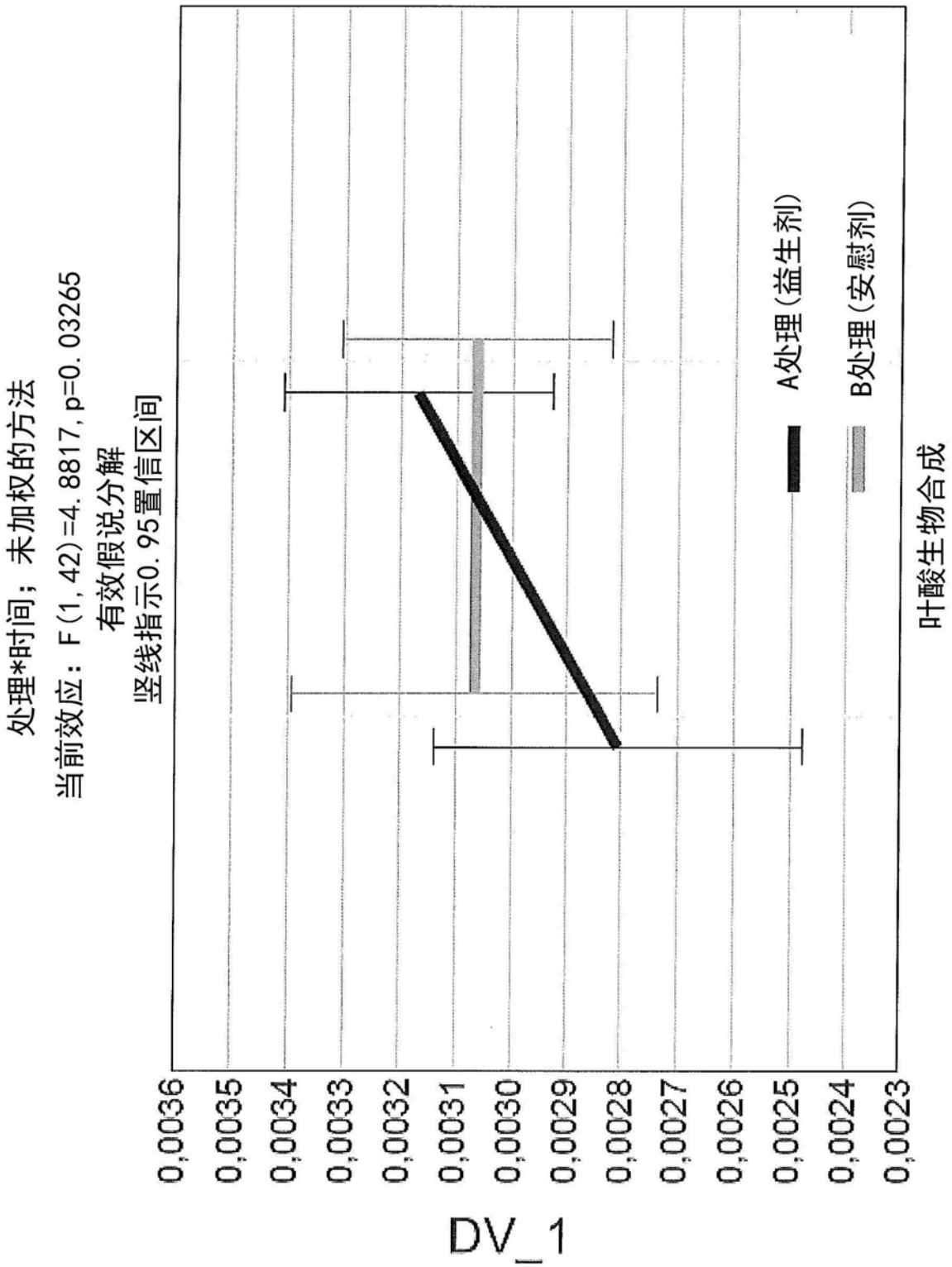

处理*时间；未加权的方法
当前效应： F(1, 42)=4. 8817, p=0. 03265
有效假说分解
竖线指示0. 95置信区间
A处理(益生剂)
B处理(安慰剂)
叶酸生物合成
0,0036
0,0035
0,0034
0,0033
0,0032
0,0031
0,0030
0,0029
0,0028
0,0027
0,0026
0,0025
0,0024
0,0023
DV_1

图4