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(54) Title: METHOD, DEVICE AND COMPUTER PROGRAM PRODUCT FOR DIAGNOSING OF AT LEAST ONE EXHAUST EMISSION CONTROL UNIT

(57) Abstract: The present invention relates to a kit comprising at least two compositions, wherein each of said compositions comprises at least one strain chosen from Lactobacillus. The Lactobacillus strains are chosen from the group consisting of Lactobacillus plantarum, lactobacillus rhamnosus, lactobacillus fermentum, lactobacillus paracasei and Lactobacillus gasseri.

KIT COMPRISING AT LEAST TWO LACTOBACILLUS COMPOSITIONS

Field of the invention

The present invention relates to a kit comprising at least two compositions, wherein each of said compositions comprises at least one strain chosen from Lactobacillus. The invention further relates to a method for administration of said kit, wherein each of said compositions of the kit is administered in a sequence or in an alternating sequence or in a combination thereof.

Background art

Probiotoic bacteria are defined as live microorganisms which when administered in adequate amounts beneficially affect the host. *Lactobacilli* and *bifidobacteria* are the most frequently used bacteria in probiotic products. These bacteria are generally safe, as are probiotics based on these organisms. The lack of pathogenicity extends across all age groups and to immunocompromised individuals. Intake of different probiotic bacteria has been shown to have clinical benefits in various physiologic or pathologic situations. The most clear cut effects have been shown in diarrhoea caused by antibiotic therapy or rotavirus infection. There are also studies showing positive clinical effects in inflammatory bowel diseases, atopic dermatitis and hypercholesterolemia after intake of probiotic bacteria. The mechanism, by which probiotic bacteria contribute to these clinical improvements are not clear. *In vitro* human, as well as both *in vivo* and *in vitro* animal studies, have shown that different species of lactobacilli affects the innate and acquired immune system in many different ways. Clinical studies have mainly shown stimulation of the innate cellular immune system and enhancement of humoral immune responses to natural infections and systemic or oral immunisation. Regarding effects of the innate immune system, increased

phagocytic activity of polymorphonuclear cells (PMN) and increased NK cell tumor killing activity have been reported. To our knowledge, there are no clinical studies showing effects on the specific cellular immune system after intake of probiotic bacteria.

In the present application the effects on the innate and acquired immune system following daily intake of *lactobacilli* or the Gram-negative bacteria *P. lundensis* have been thoroughly investigated. Interestingly, it has been observed an activation of the specific cellular immune system in subjects receiving *L. plantarum* and indications of such in subjects receiving *L. paracasei*. Moreover, immunity-enhancing effects on the innate immune system, such as expansion of the NKT cell population and increased phagocytoic activity were observed in subjects receiving different *lactobacilli* species. Intake of the Gram-negative bacteria *P. lundensis* had no effects, whatsoever, on the different immune parameters measured in this study.

Increased standard of living and improved sanitary conditions in the industrialized nations have increased life expectancy, foremost by reducing the burden of infection. However, at the same time, a number of diseases have, instead, increased. These include the three types of diseases that results from exaggerated or dysregulated immunity, namely allergies, autoimmunity and inflammatory bowel disease. All these three types of diseases are more common in developed than in developing countries, more common in small than large families and more common in urban than rural populations. This has led to the formulation of "the hygiene hypothesis", which states that the infant's developing immune system needs to obtain a certain degree of stimulation by infections, or perhaps commensal microbes, in order to mobilize regulatory T cells so that proper tolerance mechanisms can develop.

A number of measures to reduce allergies have been instituted. Parents have been given advice to avoid allergens, breast-feed and avoid smoking. Depite all attempts, allergies and other immunoregulatory diseases continue to increase globally.

In the present application a method to expand and/or increase the functional activity of regulatory T cells has been studied, thereby reducing the risk of developing immunoregulatory diseases such as allergy, autoimmune diseases (type 1-diabetes and multiple sclerosis) and inflammatory bowel disease. By intake of a sequence of lactobacillus strains, the immune system is activated in a manner that leads to formation of functionally active regulatory T cells. These cells can then down-regulate potentially harmful immune mediated diseases, such as allergy.

Summary of the invention

An object of the present invention is a kit comprising at least two compositions, wherein each of said compositions comprises at least one strain chosen from Lactobacillus.

Another object of the present invention is a method for administration of above mentioned kit, wherein each of said compositions of the kit is administered in a sequence or in an alternating sequence or in a combination thereof.

Brief description of the drawings

Figure 1 shows the numbers of volunteers reporting any minor adverse gastrointestinal effects during the trial.

Figure 2 shows base line numbers (day 0) of different lymphocytes per ml blood (mean ± (SEM))

Figure 3 shows base line (day 0) percentages or GMFI (mean ± (SEM)) of lymphocytes positive for different cell activation and memory markers.

Figure 4. Subjects were randomly assigned to nine different study groups. The trial started with a wash out

period of two weeks. Thereafter, the active study period followed. During this period, the subjects consumed one dose of study product per day for 14 (*L. Heal 19*, *L. fermentum*, *L. paracasei*, *L. gasseri*, *L. rhamnosus*, *P. lundensis* groups) or 35 days (*L. plantarum* and placebo group). Each dose contained 10^{10} coloni forming units (CFU) (*lactobacilli* groups) or 10^{9} CFU bacteria (*P. lundensis* group). Subjects were supplied with a list of probiotic products, which should not be consumed during the trial. A diary, in which each subject stated adverse effects, health conditions and confirmed intake of study product, was kept during the trial.

Figure 5. Percentages of lymphocytes expressing the activation phenotypes CD3CD8CD25, CD3CD8HLA-DR, CD3CD4CD25 and CD3CD4HLA-DR was analysed by flowcytometry. Group means (±SEM) based on individual ratios, day 14/day 0 and day 35/day 0 (for *L. plantarum* and placebo group only) is shown.

Figure 6. Percentages of lymphocytes expressing the memory phenotypes CD3CD8CD45RO and CD3CD4CD45RO was analysed by flowcytometry. Group means (±SEM) based on individual ratios, day 14/day 0 and day 35/day 0 (for *L. plantarum* and placebo group only) is shown.

Figure 7. Percentages of lymphocytes positive for the NKT cell markers (CD56CD16CD3) was analysed by flowcytometry. Group calculations are based on individual ratios (day 14/day 0).

Figure 8. The phagocytic activity of neutrophils was analysed by incubating whole blood cells with FITC-labelled E. coli or S. aureus. The ratio between mean fluorescence values obtained at day 14 and day 0 was determined individually and group calculations are shown in this figure.

Figure 9 shows the ratio of lymphocytes expressing the activation phenotypes CD4CD25 from experiment 2.

Figure 10 shows the ratio of lymphocytes expressing the activation phenotypes $CD4^{+}CD25^{++}$ from experiment 2.

Figure 11 shows the ratio of lymphocytes expressing the activation phenotypes $CD8^+HLA\text{-}DR^+$ from experiment 2.

Figure 12 shows the ratio of lymphocytes expressing the activation phenotypes CD8+CD25+ from experiment 2.

Figure 13 shows the ratio of lymphocytes expressing the activation phenotypes CD4CD45RO from experiment 2.

Detailed description of the invention

The compositions comprised in the kit according to the invention and/or the compositions according to the invention may be independently selected from food compositions, dietary supplements, functional foods, medical foods and nutritional products.

The above mentioned food compositions may e.g. be beverages, yoghurts, juices, ice creams, breads, biscuits, cereals, health bars, spreads, or nutritional products.

Preferably, the kit according to the invention comprises at least 10 of the above mentioned compositions. More preferably the kit according to the invention comprises at least 7 of the above mentioned compositions. Most preferably the kit according to the invention comprises at least 5 of the above mentioned compositions.

The Lactobacillus used according to the invention may be selected from, but not limited to, the group consisting of *Lactobacillus plantarum*, *Lactobacillus rhamnsosus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus gasseri*.

The *Lactobacillus plantarum* used according to the invention may be selected from, but not limited to, the group consisting of *Lactobacillus plantarum* 299, DSM 6595, *Lactobacillus plantarum* 299v, DSM 9843, *Lactobacillus plantarum* HEAL 9, DSM 15312, *Lactobacillus plantarum* HEAL 19, DSM 15313, and *Lactobacillus plantarum* HEAL 99, DSM 15316.

The *Lactobacillus paracasei* used according to the invention may be selected from, but not limited to, the

group consisting of *Lactobacillus paracasei* 8700:2, DSM 13434, and *Lactobacillus paracasei* 02A, DSM13432.

The Lactobacillus gasseri used according to the invention is selected from, but not limited to, Lactobacillus gasseri VPG44, DSM 16737.

Other probiotic bacterial strains, than the ones explicitly disclosed herein, may naturally be used in the present invention and are comprised of the invention.

Preferably, the compositions of the kit according to the invention and/or the composition according to the invention further comprise a carrier material. Said carrier material may independently be selected from the group consisting of oat meal gruel, lactic acid fermented foods, resistant starch, dietary fibres, carbohydrates, proteins, and glycosylated proteins.

Preferably, said at least one strain in the compositions of the kit according to the invention is present in an amount from about $1x10^6$ to about $1x10^{14}$ CFU. More preferred is that said at least one strain in the compositions of the kit according to the invention is present in an amount from $1x10^8$ to $1x10^{12}$. Even more preferred is that that said at least one strain in the compositions of the kit according to the invention is present in an amount from $1x10^9$ to $1x10^{11}$.

Preferably, said at least two strains in the composition according to the invention are each present in an amount from about $1x10^6$ to about $1x10^{14}$ CFU. More preferred is that said at least two strains in the composition according to the invention are each present in an amount from $1x10^8$ to $1x10^{12}$. Even more preferred is that that said at least two strains in the composition according to the invention are each present in an amount from $1x10^9$ to $1x10^{11}$. It is also possible that the at least two strains are present in a total amount from about $1x10^6$ to about $1x10^{14}$ CFU, preferably $1x10^8$ to $1x10^{12}$, and more preferably $1x10^9$ to $1x10^{11}$.

The kit according to the invention may be administered in a sequence or in an alternating sequence or in a combination thereof. This means that the compositions comprising the respective strain or strains could be administered alternating, e.g. strain A is administered first, then strain B is administered and then strain C is administered. Thereafter the sequence is repeated. It is also possible that first an alternating strain is administered (i.e. first A and then B and C) and thereafter only one certain strain is administered, e.g. strain D, and thereafter another or the same sequence of bacterial strains are administered. The compositions of the kit may also be administered simultaneously.

Preferably, the kit is administered in a way such that each composition of the kit is administered at time intervals of between approximately 12 h and approximately 14 days, preferably approximately 24 h and approximately 7 days, more preferably between 24 h and 48 h. In one embodiment of the invention one composition comprising at least one probiotic bacterial strain is administered per day.

According to the invention it is possible to prevent virus infections by giving a kit comprising the compositions according to the invention to an individual. Treatable virus infections are i.e. those caused by a virus selected from the group consisting of common cold virus, rhinovirus, adenovirus, parainfluenza virus, respiratory syncytial virus, enterovirus and corona virus.

Thus, the use of a kit according to the invention may be very beneficial in the sense of being usable prophylactically, i.e. before the virus infection has developed. It is for instance very convenient for a normal healthy individual to take to composition of the invention prophylactically to stay healthy.

Examples

Example 1

Subjects and trial criteria

Fifty-seven apparently healthy volunteers within the age range 18-55 years (median, 26 years) were selected for this blind placebo controlled study. Subjects were randomly assigned to eight groups, receiving either one of the following Gram-positive bacteria, L. plantarum 299v (n=7), L. plantarum Heal 19 (n=7), L. fermentum 35D (n=7), L. paracasei 8700:2 (n=7), L. gasseri VPG44 (n=7), L. rhamnosus 271 (n=7), or the Gram-negative bacteria, P. lundensis (n=7) or placebo (n=10). The dose of bacteria was 10^{10} bacteria/day for lactobacilli and 10^{9} bacteria/day for P. lundensis. The control group took skim milk powder (1 g). Depending on the group, the study had a duration period of 6 or 9 weeks consisting of two weeks wash out period, 2 or 5 weeks active study period and 2 weeks follow up period (Fig. 4). Each subject was supplied with a list of products containing probiotic products, which should not be consumed during the whole study period. Peripheral blood samples were withdrawn from subjects by venipuncture at two or three time points, day 0, day 14 and day 35. A diary, in which each subject stated adverse effects, health conditions and confirmed intake of study product, was kept during the trial.

Flow cytometry

Phenotypic analysis of lymphocytes in whole blood was performed by flow cytometry. The following anti-human monoclonal antibodies were used as surface markers for different cell populations: CD3 FITC (SK7), CD4 APC (SK3), CD8 PerCP (SK1), CD19 PerCP (SJ25C1), CD56 PE (MY31), CD16 PE (B73.1), and CD5 FITC (L17F12). Following anti-human monoclonal antibodies were used for detection of different activation and memory markers: CD25 FITC (2A3), HLA-DR PE (L243), CD45RO PE (UCHL-1), CD38 PE (HB7), CD27 PE (L128), and CD11b PE (D12). All antibodies were purchased from Becton-Dickinson (Erembodegum,

Belgium). Whole blood (100 µl) was incubated with antibodies (10 µl/antibody) for 30 min at 4° C in the dark. Thereafter, 2 ml of FACS lysing solution (Becton-Dickinson) was added and incubated for 15 min at 20° C in the dark. Cells were washed by adding 3 ml FACSFlow and centrifuged at 300 x g for 5 min. Washed cells were resuspended in 200 µl FACSFlow and analysed on a FacsCalibur (Becton-Dickinson) with CellQuest software.

Phagocytosis assay

The phagocytic activity of granulocytes and monocytes were quantified with PHAGOTEST® (Orpegen Pharma, Heidelberg, Germany) according to manufacturers instruction with some modifications. Briefly, 20 $x10^6$ FITC labelled *E. coli* or FITC labelled *S. aureus* was added to pre-cooled whole blood (100 µl). Blood cells and bacteria were incubated on 37° C for 10 FacsCalibur with CellQuest software.

Calculations

Individual changes regarding different immune parameters were determined by calculating the ratio between the individual values obtained at day 14 and day 0, or the values at day 35 and day 0. These ratios were used for all group calculations and statistics.

Statistics

All statistical analyses were performed using Statview. Mann-Whitney U test were used to compare different groups.

Results

Clinical observations

Fifty-four out of fifty-seven volunteers completed the study. Two persons were excluded due to infection and antibiotic treatment (one in the placebo group and one in the group receiving *P. lundensis*). One person was excluded day 16 due to pregnancy (placebo group). Only mild adverse gastrointestinal side effects were reported following intake of study products (Fig. 1).

Intake of lactobacilli activates T cells

There were great baseline (day 0) individual variations regarding activation markers on $CD4^+$ and $CD8^+$ T cells. The baseline percentages of cells expressing different cell surface markers are shown in Fig. 2. No significant differences were observed between different groups at this time point. Since huge inter-individual variations were observed, it was chosen to compare ratio values at day 14 and day 35 compared to day 0 for each individual. All calculations and comparisons were done on these ratio values (day 14/day 0 and day 35/day 0). After 14 days of intake of study product containing *L. plantarum* 299v there was an approximately twofold increase of the expression of the activation marker CD25 on $CD8^+$ T cells ($p=0.01$) (fig. 5). There was also a strong, although not significant ($p=0.12$), indication of upregulation of HLA-DR on $CD8^+$ cells following *L. plantarum* 299v intake. In addition, it was also observed a tendency towards activation of $CD4^+$ T cells after *L. plantarum* 299v intake. Intake of the other *lactobacilli* species included in this study, as well as the Gram-negative bacteria *P. lundensis* activated neither $CD8^+$ nor $CD4^+$ T cells. However, there was a tendency that intake of *L. paracasei* did increase the expression of HLA-DR on $CD4^+$ T cells ($p=0.18$).

Intake of lactobacilli induces a memory phenotype of CD4+ T cells

Geometric means of the fluorescence intensity (GMFI) of the expression of CD45RO on CD4+ and CD8+ T cells were compared between groups receiving different study products. As above, group calculations based on individual ratio values (day 14/day 0 and day 35/day 0) were used for comparisons. After 35 days of intake of study product containing *L. plantarum 299v* the CD45RO GMFI on CD4+ T cells increased significantly ($p=0.03$). There was also a tendency towards increased CD45RO expression on CD8+ T cells following intake of *L.*

plantarum (Fig. 6). Moreover, intake of *L. paracasei* seems to have a positive effect on upregulation of CD45RO on CD8+ T cells (p=0.10) (Fig. 6).

Effect on different cell populations following intake of study product

Following intake of *L. pararcasei* there was an increase in the percentage of lymphocytes being identified as NKT cells (P=0.06) (Fig 7). Relative increase/decrease compared to day 0 could not be detected regarding other cell populations, such as CD4+ T cells, CD8+ T cells, B cells, B-1 cells (CD19+CD5+), NK cells, granulocytes and monocytes.

Phagocytic activity

Granulocytes and monocytes were identified in the FSC-SSC diagram. The ability of these cells to phagocytose FITC-labelled Gram-positive or Gram-negative bacteria was tested. As shown in fig. 8, granulocytes from volunteers given *L. plantarum* 299v (p=0.064), *L. plantarum Heal 19* (p=0.064), *L. fermentum* (p=0.064) or *L. paracasei* (p=0.05) were more efficient then granulocytes from placebo treated volunteers in phagocytosis of the Gram-negative bacteria *E. coli*. However, there was no difference between the groups in phagocytosis of the Gram-positive bacteria *S. aureus*. No differences in the phagocytic activity of monocytes could be detected (data not shown).

Discussion

The primary task of the immune system is to react rapidly and violently to micro-organisms thereby preventing and curing infections. The killing of microorganisms employs powerful mechanisms that also cause harm to our own tissues. Therefore, it is necessary that it neither reacts to our own tissues, nor to innocuous substances present in the environment. Therefore, the immune system develops and maintains tolerance both to the components of our own body, and to food and inhaled proteins. If this fails, a number of

diseases may arise. Means to develop specific immune tolerance are an essential task of the immune system.

A central role in all immune reactions is played by the T helper cell. When a T helper cell becomes activated by its specific antigen, it becomes activated, divides, matures and produces a range of cytokines which direct the action of other types of cells in the immune system, such as cytotoxic T cells and B cells. Activation of T helper cells is necessary in order to produce most types of immune reactions, including production of antibodies. Conversely, if activation of T helper cells is prevented, most types of immune reactions are paralysed.

There are several mechanisms by which activation of T helper cells and maintenance of tolerance is ensured. One mechanism is elimination in the thymus of T cells with capacity to recognize and react to own tissue. However, this elimination is not complete and, furthermore, we also need to develop specific immune tolerance to exogenous antigens. Otherwise we would react violently to all types of inhaled and ingested substances, leading to massive inflammation and wasted immune resources.

A cell type that is central for maintenance of tolerance is the regulatory T cell. This cell type can be recognized by certain markers, such as surface expression of CD4 and CD25, possession of intracellular CTLA-4, and transcription of the nuclear protein Foxp3. The regulatory T cells are capable of preventing other T cells to become activated when encountering harmless substances and, hence, prevent all types of unwanted immune reactions.

In the present context the symbol "+" in connection with a certain marker such as CD4+ and CD25+ means that the marker is expressed on a T cell. For instance CD4+CD25+ T cells are T cells that expresss both the CD4 marker and CD25 marker on its surface. However, nothing is said about the amount of the marker that is expressed,

only that it is present. In the present context the symbol "++" in connection with a marker such as CD4++ or CD25++ means that there is a lot of marker expressed. The regulatory T cells are those cells with a lot of CD25 on the surface, i.e. CD4+CD25++ cells. On the other hand, CD4+CD25+ T cells are only activated T cells. Sometimes the specific symbols "+" and "++" are not used, e.g. CD4CD25 only, and this means that the cells are activated such CD4+CD25+ cells. Thus, CD4CD25 is the same as CD4+CD25+. When discussing regulatory T cells, it is always written as CD4+CD25++ cells.

This blind placebo-controlled study is unique in that it is the first study comparing the influence of several immune parameters following intake of different Gram-positive *lactobacilli* or the Gram-negative bacteria *P. lundensis*. Interestingly, intake of *P. lundensis* did not influence any of the measured parameters. In contrast, intake of *lactobacilli* affected different components of both the specific and innate immune system. A novel finding in this study was that intake of *L. plantarum* had a pronounced positive effect on activation and induction of memory cells in the T cell populations. There was a significant upregulation of the IL-2 receptor α chain (CD25) and a strong tendency towards upregulation of HLA-DR on cytotoxic T cells. A tendency towards upregulation of these activation markers was also observed on helper T cells after intake of *L. plantarum*. Expression of activation markers indicates that the T cells have started to proliferate in response to antigen-specific or non-specific stimuli and that these cells more readily exert their effector functions compared to resting T cells. The mechanisms behind *L. plantarum* induced activation of T cells could be via antigen presenting cells that are activated by toll-like receptors binding to microbial compounds. Activation of antigen presenting cells makes them more efficient in presenting antigen to T cells. In addition, both helper

and cytotoxic T cells have shown to have various expressions of toll-like receptors, which probably make these cells sensible for non-specific activation by microbial components and products.

In analogy to the helper T cell compartment, expression of CD45RO seems to mark a memory population also among cytotoxic T cells. There was found a significant increase in the expression of this memory cell marker on helper T cells, and a tendency towards upregulation on cytotoxic T cells following 35 days intake of *L. plantarum*. In addition, intake of *L. paracasei* also showed a tendency towards upregulation of CD45RO on cytotoxic T cells. Relative to naïve T cells, CD45RO+ T cells can secrete a broad spectrum of cytokines. Moreover, CD45RO+ T cells can proliferate and produce IL-2 when the CD3-TCR complex is stimulated under suboptimal conditions, whereas naïve T cells require a strong CD3-TCR stimulus to carry out these functions. The formation of memory T cells is important for induction of an efficient immune response after infection and vaccination.

The innate cellular immune system was also affected by intake of probiotic bacteria. It was demonstrated that the natural killer T (NKT) cell population was expanded following intake of *L. paracasei*. NKT cells constitute a lymphocyte subpopulation that coexpress the NK cell marker CD56 and the T cell marker CD3-T cell receptor complex. Studies in both humans and mice have demonstrated that NKT cells play a central role in the regulation of autoimmune diseases, such as multiple sclerosis, type I diabetes, and systemic lupus. NKT cells also exert effector functions against tumour and virus infected cells. Thus, NKT cells are pleotropic in their functions. Other clinical studies evaluating the immunological effects of probiotic bacteria have shown that intake of *L. rhamnosus* HN001 and *Bifidobacterium lactis* HN019 enhance NK (including NKT) cell tumour

killing activity of K562 cells. In this study it was also confirmed the observation by others that phagocytic activity of polymorphonuclear cells is increased after intake of different lactobacilli. The consequence of the observed effects on the different immune parameters in the present study is that one could speculate that the coincident activation of cytotoxic T cells and NKT cell expansion points to a strengthened immune defense against viral infections and/or tumours. The *in vitro* finding that lactobacilli induce mononuclear cells to secrete IL-12 and IL-18, supports the theory that intake of these bacteria stimulates cell-mediated activity.

In accordance with the present invention it has been concluded that intake of *L. plantarum* and *L. paracasei* has a profound effect on the specific and innate cellular immune system. However, the increase in immune function demonstrated herein is for the time being difficult to correlate to a proven health benefit in humans. In order to address this specific issue, further clinical trials in individuals suffering from e.g. viral infections or tumours need to be accomplished. In such studies, it would be of special interest to compare the effect of administration of *L. plantarum* and *L. paracasei* separately or in combination.

Example 2

The goal of this example was to investigate the effect on the immune system by giving the same species of lactobacilli for a longer period of time compared to several lactobacilli (different species) administered in a sequence one after the other.

The volunteers were given a powder with freeze-dried bacteria during 14 or 35 days. As gram-positive bacteria the probiotic bacteria Lactobacillus plantarum 299v is used alone or in combination with L. rhamnosus, L. fermentum, L. paracasei, and L. gasseri. As gram-negative bacteria Psedomonas lundensis is given.

The following groups are studied:
1) *Lactobacillus plantarum* 35 days
2) *L. plantarum* 7d, *L. rhamnosus* 7d, *L. fermentum* 7d, *L paracasei* 7d, *L. gasseri* 7d. Totally 35 days. (Sequence)
3) A mixture of L. *plantarum, L. rhamnosus, L. fermentum, L. paracasei, L. gasseri.* Totally 14 days
4) *L. rhamnosus* 14 days
5) *L. fermentum* 14 days
6) *L. paracasei* 14 days
7) *L. gasseri* 14 days
8) *Pseudomonas lundensis* 14 days
Control group 1) Placebo 35 days
Control group 2) Placebo 14 days

Blood samples are taken at day 0, 14 and 35. The amount of helper T cells (CD4+) expressing high amounts of CD25 was defined in each group by flow cytometry as have been explained above in experiment 1.

Results

On day 14, there was a borderline significance of CD4+CD25++ T cells being expanded in individuals consuming the sequence of five different lactobacilli strains.

Discussion

T helper cells (CD4+) expressing high density of the CD25 molecule (CD4+CD25++) have been shown to be important in order to protect against autoimmune diseases, allergies and inflammatory bowel diseases. The finding that these cells are expanded after intake of a sequence of different lactobacilli indicate that intake of these bacteria might be beneficial for the individual concerning the risk of developing the above mentioned diseases.

CLAIMS

1. A kit comprising at least two compositions, wherein each of said compositions comprises at least one strain chosen from Lactobacillus.

2. A kit according to claim 1, wherein said composition is chosen from the group consisting of a food composition, a dietary supplement, a functional food, a medical food and a nutritional product.

3. A kit according to claim 2, wherein said food composition is chosen from the group consisting of beverages, yoghurts, juices, ice creams, breads, biscuits, cereals, health bars, spreads, and nutritional products.

4. A kit according to any one of claims 1-3, wherein said kit comprises at least 10 compositions, preferably at least 7 compositions, and most preferably at least 5 compositions.

5. A kit according to any one of claims 1-4, wherein said Lactobacillus strain is chosen from the group consisting of *Lactobacillus plantarum*, *Lactobacillus rhamnsosus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus gasseri*.

6. A kit according to claim 5, wherein said *Lactobacillus plantarum* is chosen from the group consisting of *Lactobacillus plantarum* 299, DSM 6595, *Lactobacillus plantarum* 299v, DSM 9843, *Lactobacillus plantarum* HEAL 9, DSM 15312, *Lactobacillus plantarum* HEAL 19, DSM 15313, and *Lactobacillus plantarum* HEAL 99, DSM 15316.

7. A kit according to claim 5, wherein said *Lactobacillus paracasei* is chosen from the group consisting of *Lactobacillus paracasei* 8700:2, DSM 13434, and *Lactobacillus paracasei* 02A, DSM13432.

8. A kit according to claim 5, wherein said Lactobacillus gasseri is selected from Lactobacillus gasseri VPG44, DSM 16737.

9. A kit according to any one of claims 1-8, wherein said at least two compositions comprise a carrier material.

10. A kit according to claim 9, wherein said carrier material is chosen from the group consisting of oat meal gruel, lactic acid fermented foods, resistant starch, dietary fibres, carbohydrates, proteins, and glycosylated proteins.

11. A kit according to any one of claims 1-10, wherein each of said at least two strains in the composition is present in an amount from about $1x10^6$ to about $1x10^{14}$ CFU, preferably from $1x10^8$ to $1x10^{12}$, and more preferably from $1x10^9$ to $1x10^{11}$.

12. A method for administration of a kit according to any one of claims 1-11, wherein each of said compositions of the kit is administered in a sequence or in an alternating sequence or in a combination thereof.

13. A method according to claim 12, wherein each composition is administered at time intervals of between about 12 h and about 14 days, preferably about 24 h and about 7 days, more preferably between about 24 h and about 48 h.

14. A method for administration of a kit according to any one of claims 1-11, wherein said compositions are administered simultaneously.

Fig. 1

	Wash out period (Week)		Study period (Week)					Post study period (Week)	
	-2	-1	1	2	3	4	5	+1	+2
L. plantarum	0/7	1/7	3/7	2/7	3/7	2/7	1/7	1/7	0/7
L. Heal 19	0/7	1/7	1/7	2/7				2/7	1/7
L. fermentum	0/7	0/7	0/7	0/7				1/7	0/7
L. paracasei	0/7	0/7	1/7	0/7				0/7	0/7
L. gasseri	0/7	0/7	3/7	1/7				4/7	0/7
L. rhamnosus	1/7	1/7	0/7	0/7				1/7	0/7
P. lundensis	1/6	1/6	1/6	1/6				0/6	0/6
Placebo	0/9	0/9	2/9	3/9	1/8	1/8	0/8	0/8	0/8

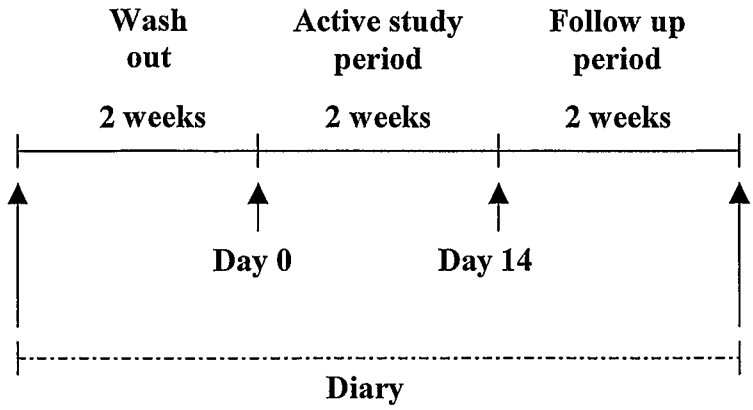
Fig. 2.

	CD4+ T cells ($x 10^3$)	CD8+ T cells ($x 10^3$)	NKT cells ($x 10^3$)
L. plantarum	647 (92)	318 (37)	64 (17)
L. Heal 19	817 (105)	328 (43)	56 (19)
L. fermentum	907 (82)	479 (51)	87 (21)
L. paracasei	794 (87)	321 (64)	98 (21)
L. gasseri	767 (54)	497 (110)	111 (39)
L. rhamnosus	775 (109)	387 (50)	109 (22)
P. lundensis	731 (65)	468 (84)	87 (29)
Placebo	650 (43)	300 (34)	107 (30)

Figure 3.

	% CD4+CD25+ of lymphocytes	% CD8+CD25+ of lymphocytes	% CD4+HLA-DR+ of lymphocytes	% CD8+HLA-DR+ of lymphocytes	GMFI CD45RO on CD4+ T cells	GMFI CD45RO on CD8+ T cells
L. plantarum	10 (0,90)	0,83 (0,19)	4,3 (0,69)	5,0 (1,8)	53 (10)	27 (5,6)
L. Heal 19	17 (2,6)	1,5 (0,40)	4,4 (1,2)	6,6 (3,3)	126 (39)	61 (15)
L. fermentum	15 (0,98)	1,5 (0,31)	4,4 (0,51)	7,0 (1,1)	71 (13)	36 (5,6)
L. paracasei	17 (1,1)	1,7 (0,73)	8,5 (4,7)	6,1 (1,6)	83 (13)	50 (17)
L. gasseri	15 (2,0)	1,3 (0,26)	3,3 (0,60)	5,2 (1,3)	110 (96)	45 (14)
L. rhamnosus	14 (0,60)	1,3 (0,13)	3,0 (0,34)	5,2 (1,3)	80 (24)	40 (11)
P. lundensis	18 (4,1)	4,0 (2,3)	10 (6,7)	9,4 (2,4)	65 (9,4)	38 (3,5)
Placebo	13 (1,0)	2,6 (1,4)	4,2 (0,59)	6,8 (3,5)	39 (8,2)	23 (5,1)

A


Wash out
2 weeks
Active study period
2 weeks
Follow up period
2 weeks
Day 0
Day 14
Diary

B

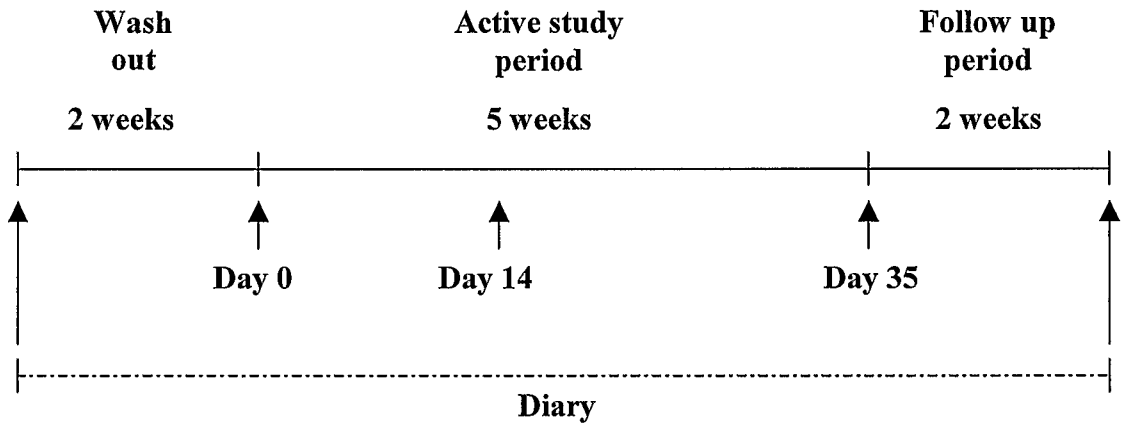
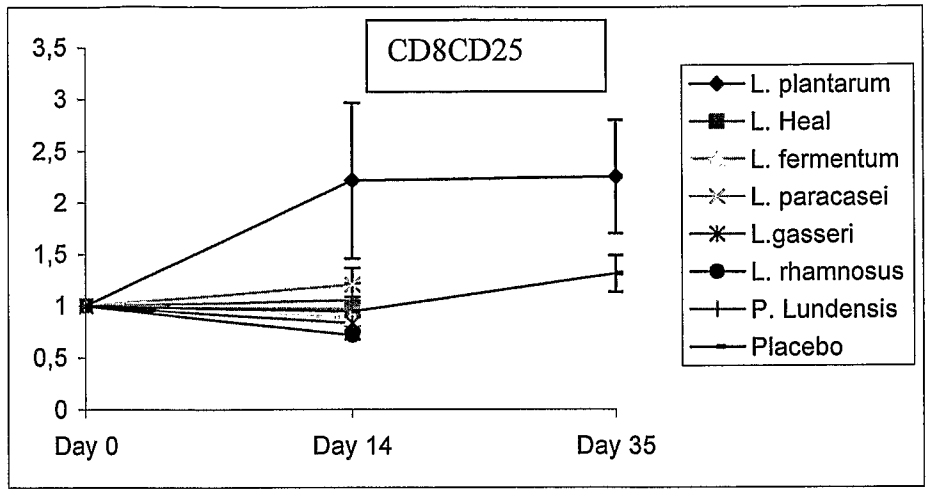
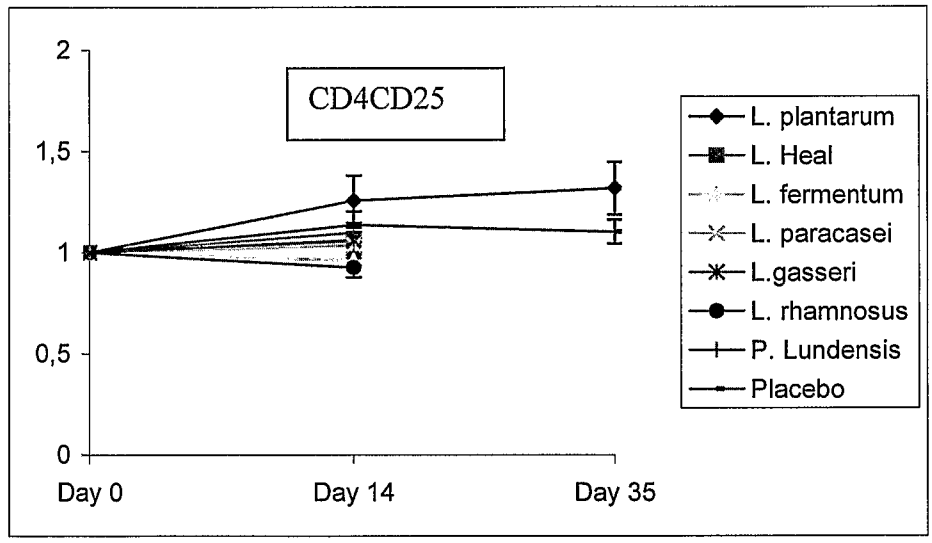

Wash out
2 weeks
Active study period
5 weeks
Follow up period
2 weeks
Day 0
Day 14
Day 35
Diary

Fig. 4

Fig. 5


CD8CD25
3,5
3
2,5
2
1,5
1
0,5
0
Day 0
Day 14
Day 35
L. plantarum
L. Heal
L. fermentum
L. paracasei
L.gasseri
L. rhamnosus
P. Lundensis
Placebo


CD4CD25
2
1,5
1
0,5
0
Day 0
Day 14
Day 35
L. plantarum
L. Heal
L. fermentum
L. paracasei
L.gasseri
L. rhamnosus
P. Lundensis
Placebo

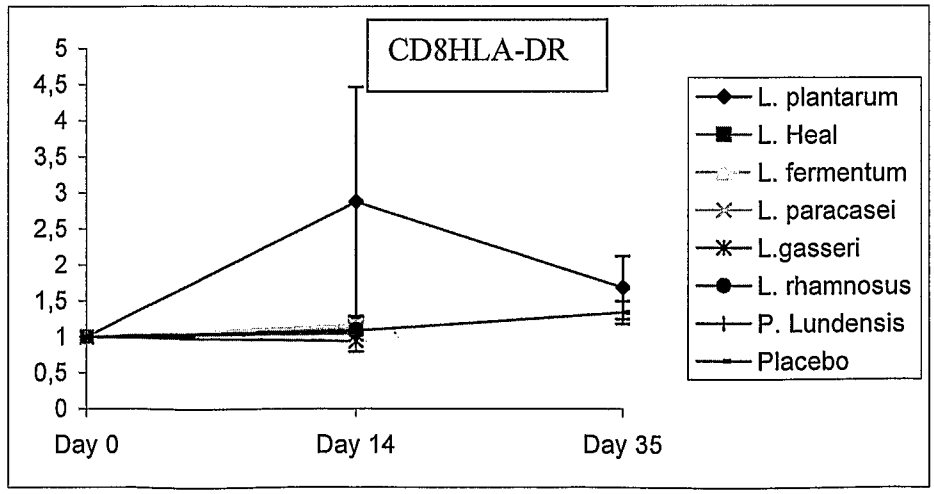

CD8HLA-DR
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Day 0
Day 14
Day 35
L. plantarum
L. Heal
L. fermentum
L. paracasei
L.gasseri
L. rhamnosus
P. Lundensis
Placebo

Fig. 5 (continued)

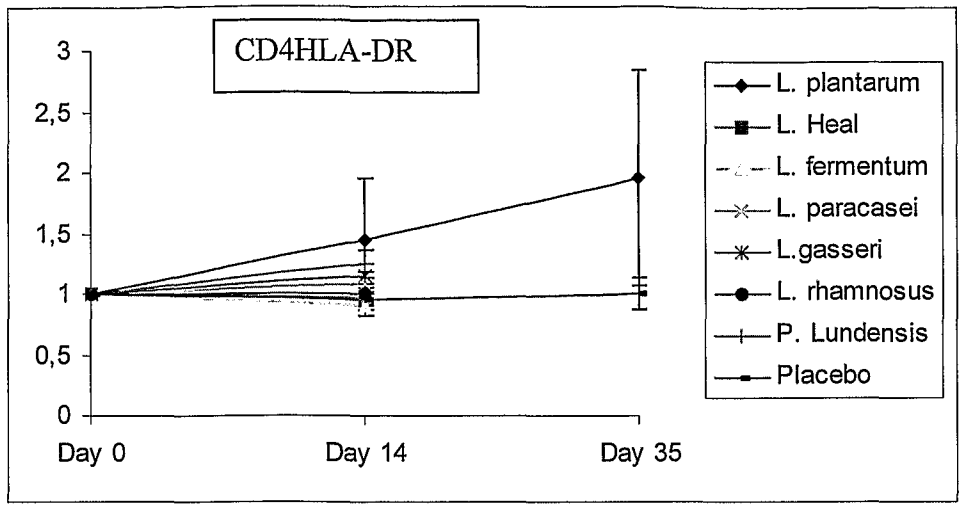
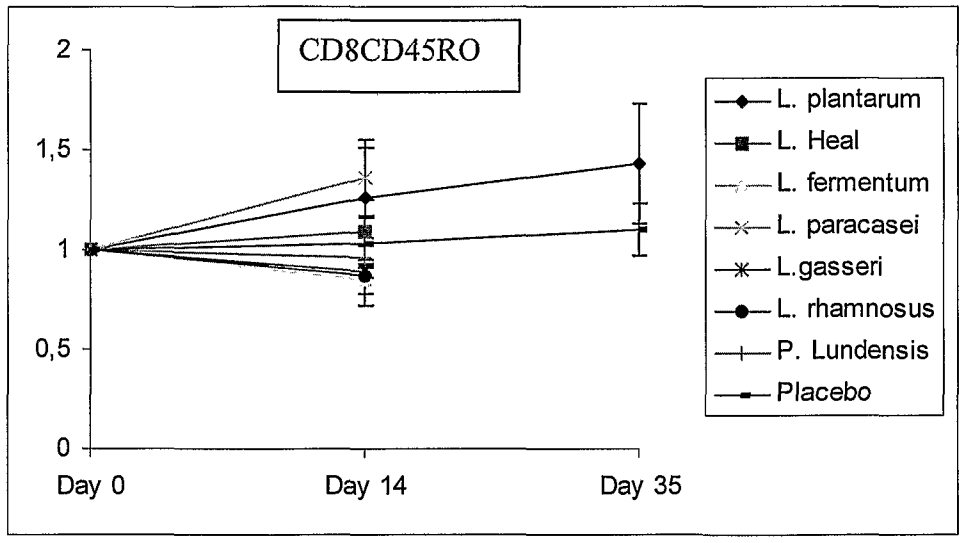
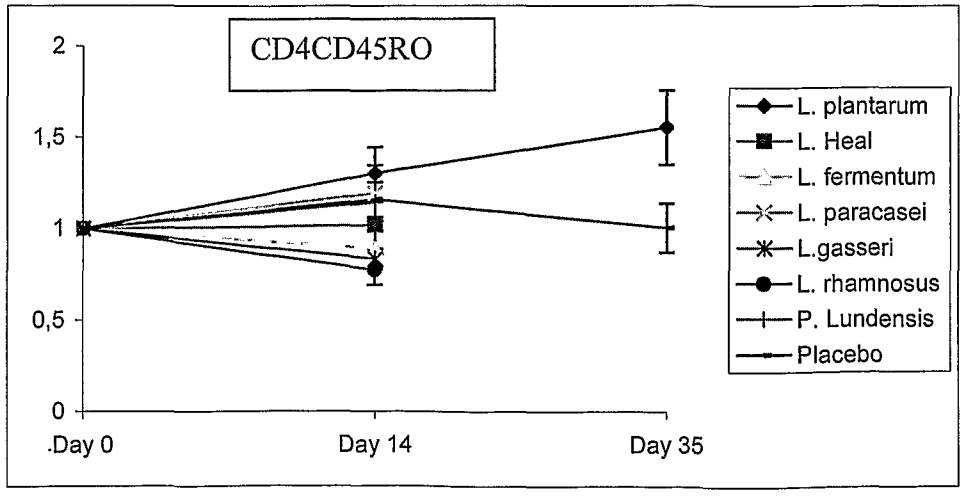


Fig. 6.





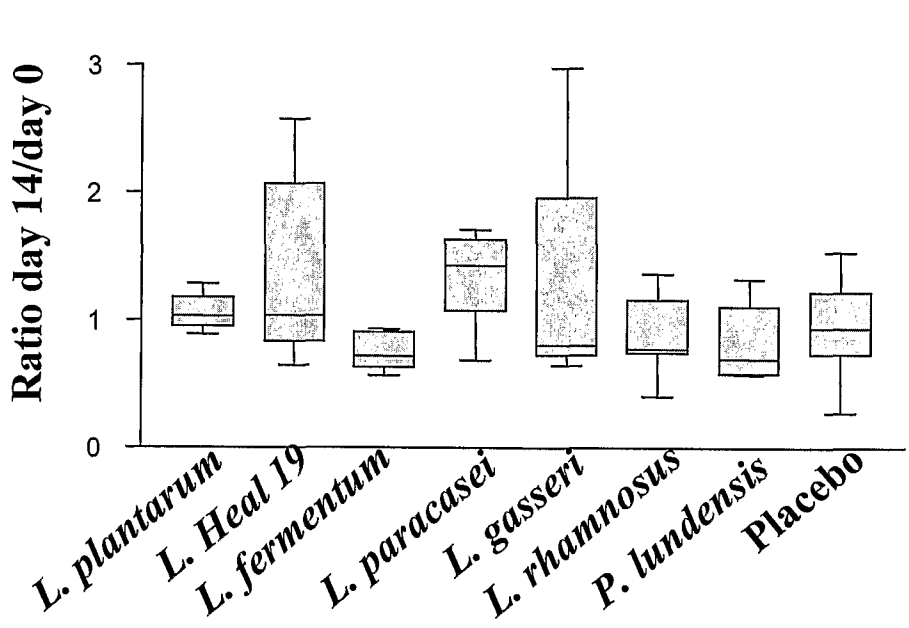

NKT cells
Ratio day 14/day 0
3
2
1
0
L. plantarum
L. Heal 19
L. fermentum
L. paracasei
L. gasseri
L. rhamnosus
P. lundensis
Placebo

Fig. 7

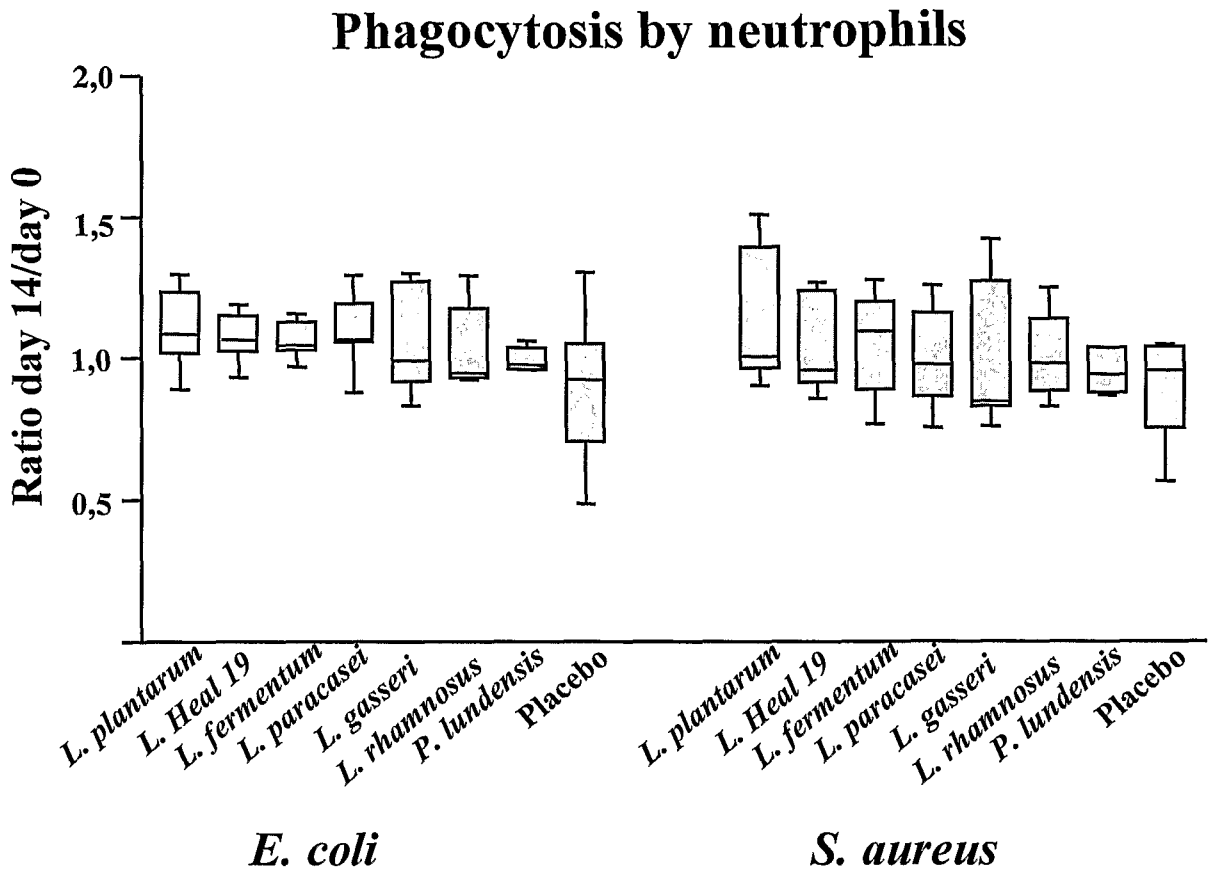

Phagocytosis by neutrophils
Ratio day 14/day 0
2,0
1,5
1,0
0,5
L. plantarum
L. Heal 19
L. fermentum
L. paracasei
L. gasseri
L. rhamnosus
P. lundensis
Placebo
E. coli
S. aureus

Fig. 8

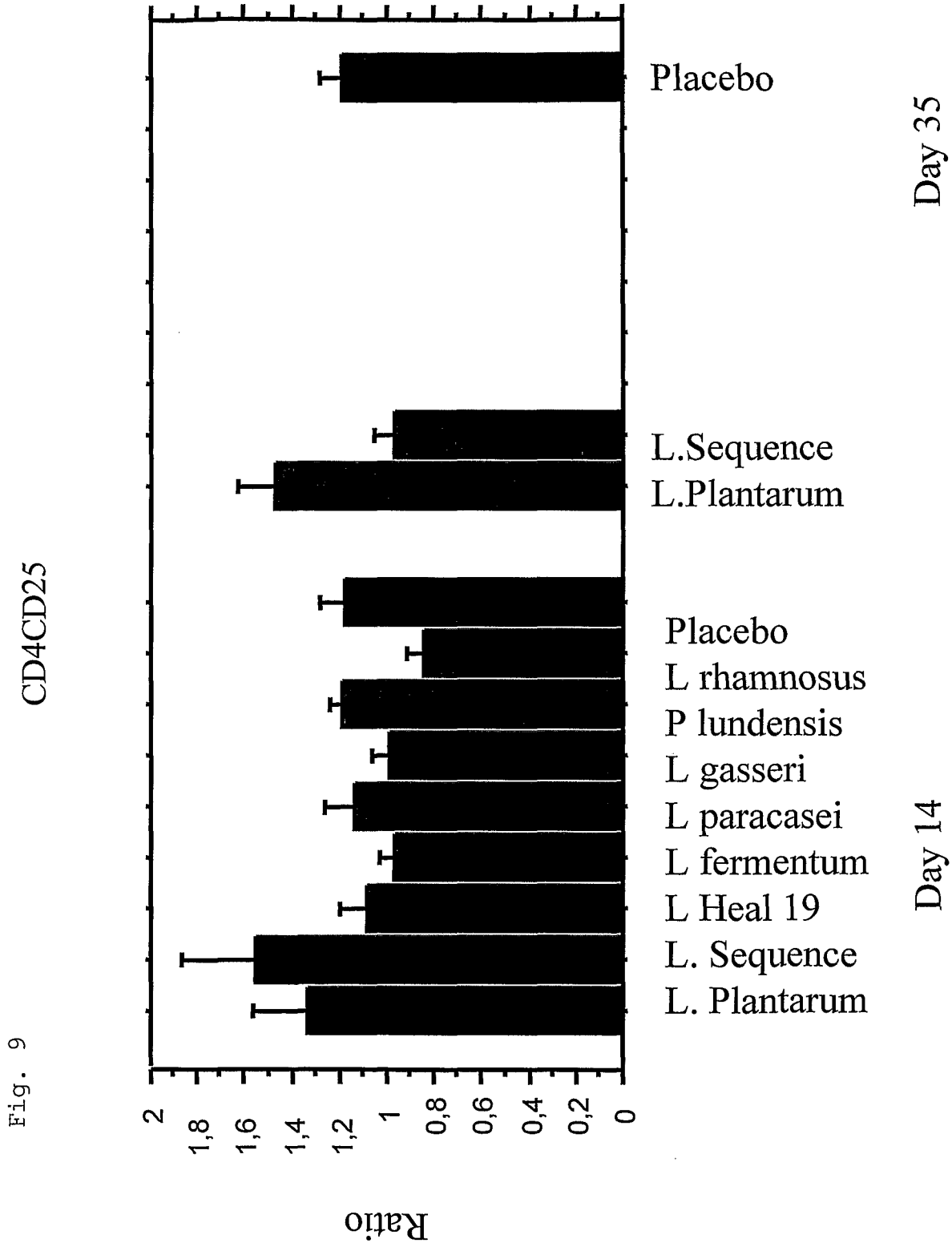

CD4CD25
Ratio
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Placebo
L.Sequence
L.Plantarum
Placebo
L rhamnosus
P lundensis
L gasseri
L paracasei
L fermentum
L Heal 19
L. Sequence
L. Plantarum
Day 35
Day 14

Fig. 9

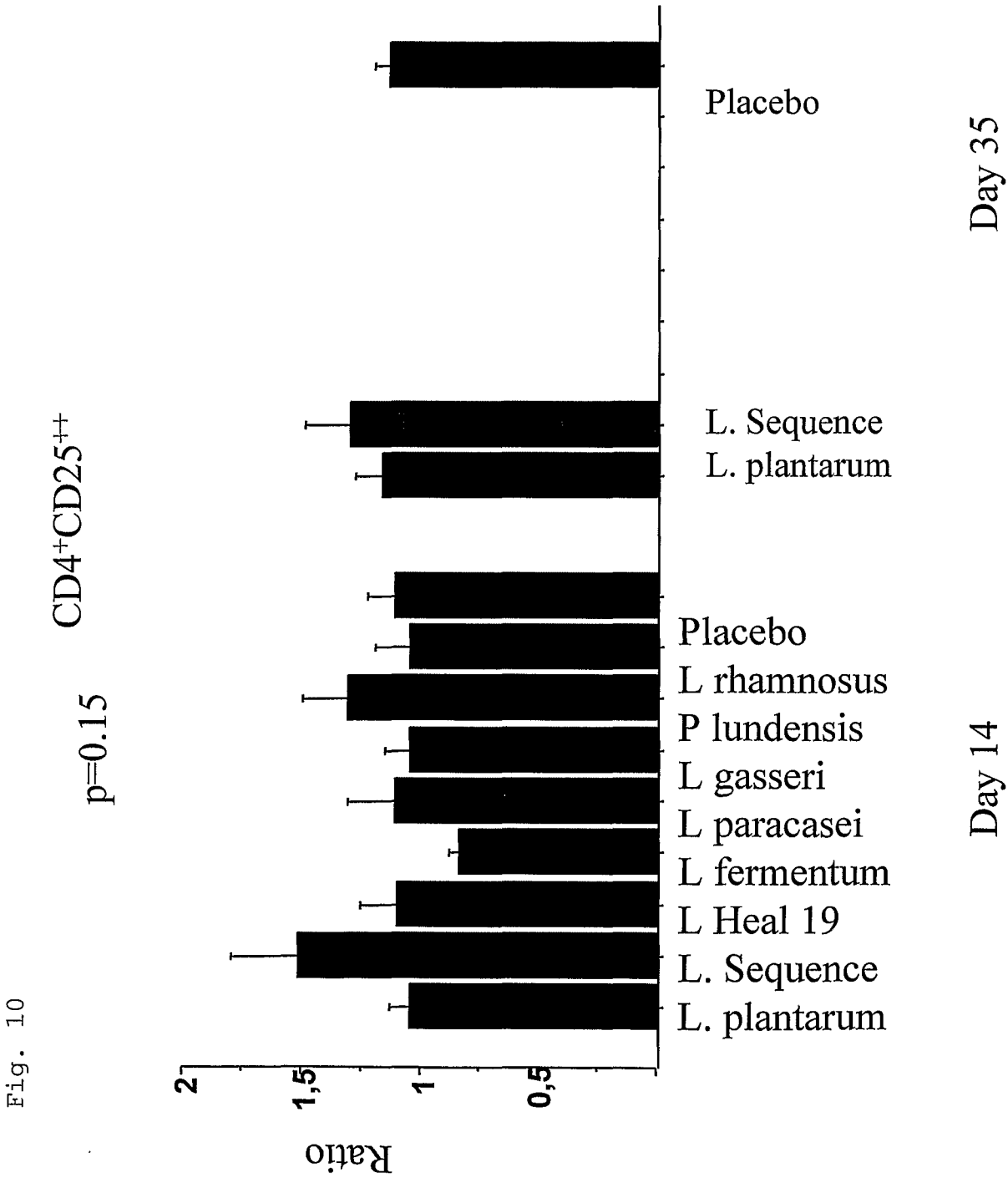

Ratio
2
1,5
1
0,5
p=0.15
CD4+CD25++
Placebo
L. Sequence
L. plantarum
Placebo
L rhamnosus
P lundensis
L gasseri
L paracasei
L fermentum
L Heal 19
L. Sequence
L. plantarum
Day 35
Day 14

Fig. 10

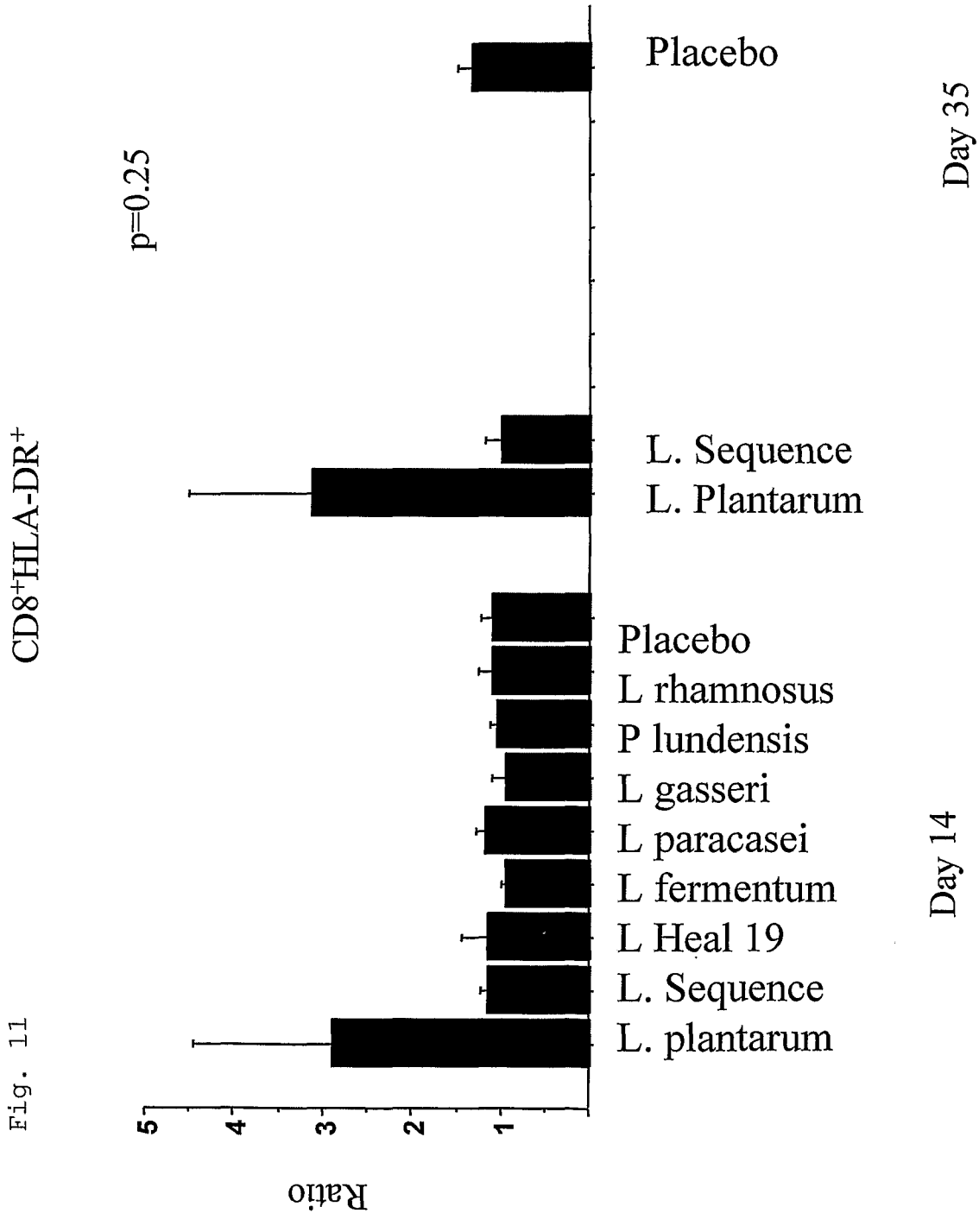

Placebo
Day 35
p=0.25
L. Sequence
L. Plantarum
CD8+HLA-DR+
Placebo
L rhamnosus
P lundensis
L gasseri
L paracasei
L fermentum
L Heal 19
L. Sequence
L. plantarum
Day 14
5
4
3
2
1
Ratio

Fig. 11

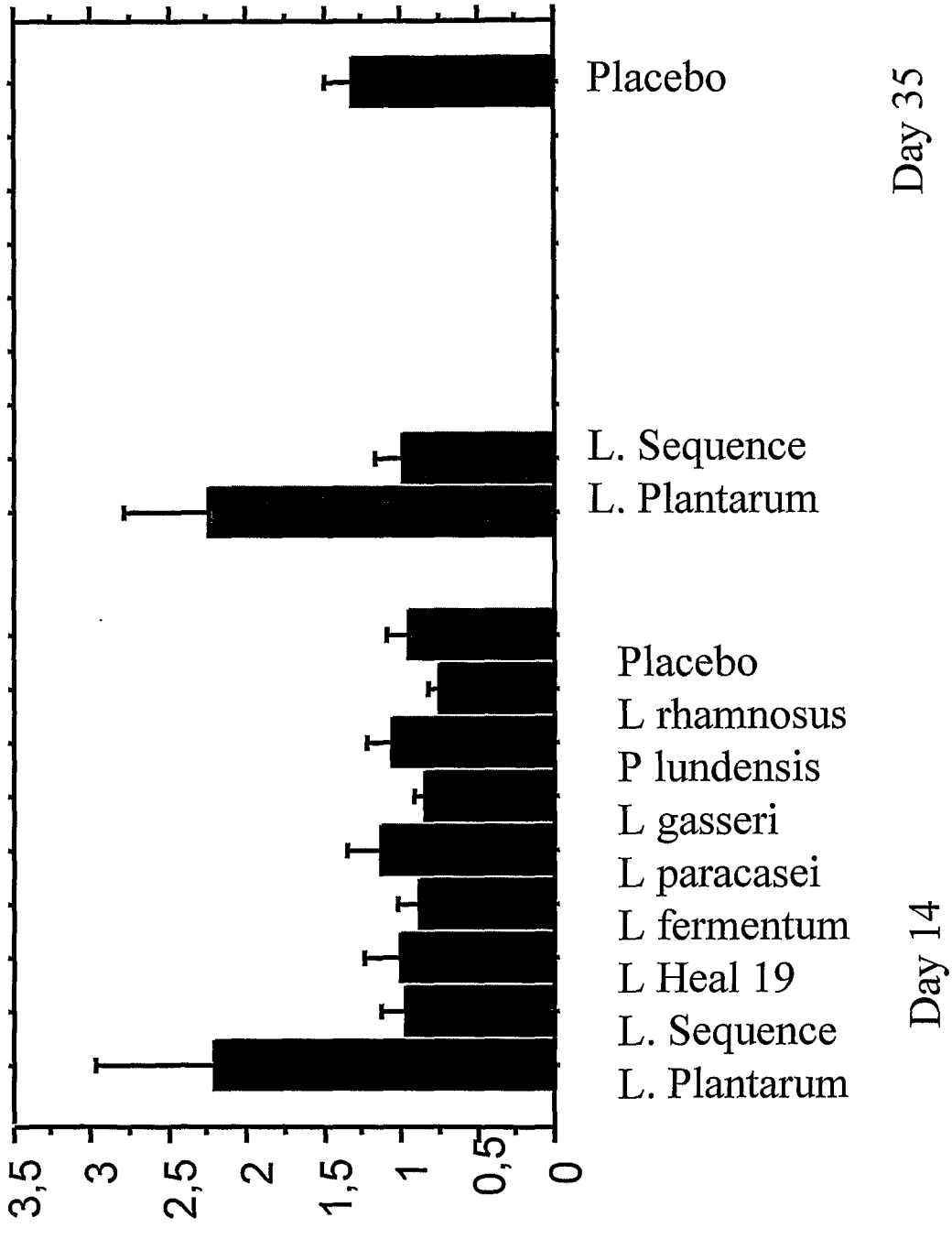

CD8+CD25+
3,5
3
2,5
2
1,5
1
0,5
0
Placebo
L. Sequence
L. Plantarum
Placebo
L rhamnosus
P lundensis
L gasseri
L paracasei
L fermentum
L Heal 19
L. Sequence
L. Plantarum
Day 35
Day 14

Fig. 12

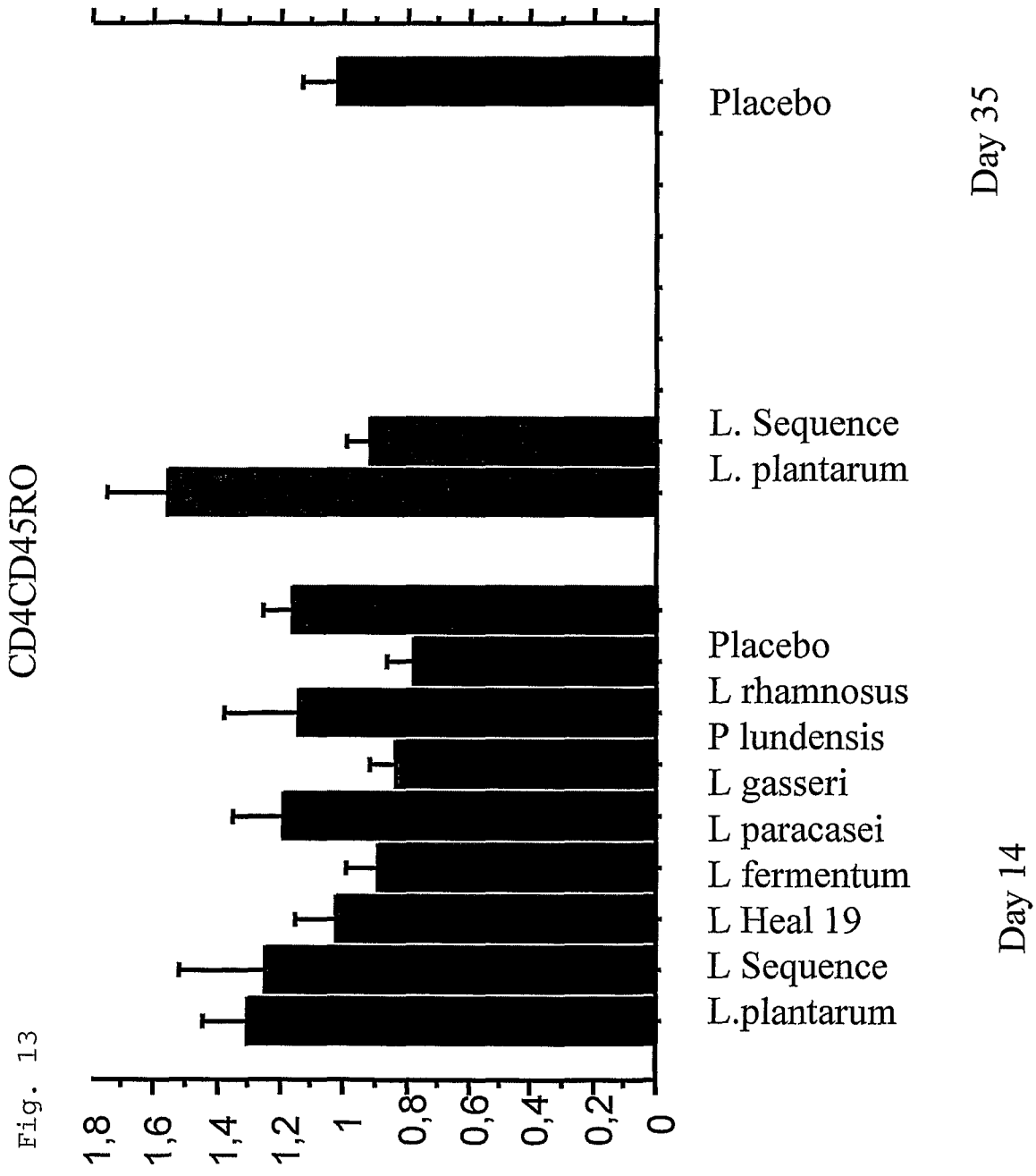

CD4CD45RO
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Placebo
L. Sequence
L. plantarum
Placebo
L rhamnosus
P lundensis
L gasseri
L paracasei
L fermentum
L Heal 19
L Sequence
L.plantarum
Day 35
Day 14

Fig. 13

Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~17~~ 5, line ~~20~~ 32.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-3300 Braunschweig

Date of deposit	Accession Number
1991-07-02	DSM 6595

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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[X] This sheet was received with the international application 30-11-2006	[ ] This sheet was received by the International Bureau on:
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Form PCT/RO/134 (July1998; reprint January 2004)

Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~eg11~~ 5, line 27 32.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-3300 Braunschweig

Date of deposit	Accession Number
1995-03-16	DSM 9843

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~eg 17~~ 5, line ~~27-28~~ 33.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2002-11-27	DSM 15312

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~eg 17~~ 5, line ~~28-29~~ 34.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution
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Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2002-11-27	DSM 15313

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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~9-17~~ 5, line ~~29-30~~ 35.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

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DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2002-11-27	DSM 15316

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2000-04-06	DSM 13434

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~9~~ 6, line ~~35~~ 2.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2000-04-06	DSM 13432

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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30-11-2006	
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Applicant's or agent's file reference PC-21028502	International application No. S2006/001137

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page ~~6~~ 6, line ~~36~~ 5.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 B
D-38124 Braunschweig

Date of deposit	Accession Number
2004-09-17	DSM 16737

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2006/001137

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
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)

IPC: A23L, A61K

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

SE,DK,FI,NO classes as above

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

EPO-INTERNAL, WPI DATA, TXTE, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0078322 A2 (MENDES S.R.L.), 28 December 2000 (28.12.2000), page 4, line 1 - line 18, claim 13, abstract	1-5,9-14
Y		6-8
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X	WO 2004103083 A1 (SYNBIOTICS AB), 2 December 2004 (02.12.2004)	1-5,9-14
A		6-8
	--	

[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
4 January 2007	18 -01- 2007
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen/EÖ Telephone No. +46 8 782 25 00

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2006/001137

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	National Library of Medicine (NLM), file Medline, Medline accession no. 10337020, Herias M V et al: "Immunomodulatory effects of colonizing the intestine of gnotobiotic rats"; & Clinical and experimental immunology, volume 116, no. 2, May 1999, page 283 - page 290	6
Y	National Library of Medicine (NLM), file Medline, Medline accession no. 16101937, Peng Guei-Cheng et al: "The efficacy and safety of heat-killed Lactobacillus for treatment of perennial allergic rhinitis induced by house-dust mite"; & Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology, vol. 16, no. 5, August 2005, page 433 - page 438	7
Y	National Library of Medicine (NLM), file Medline, Medline accession no. 12076036, Kitazawa Haruki et al: "A novel immunostimulating aspect of Lactobacillus gasseri : induction of "Gasserokine" as chemoattractants for macrophages"; & International journal of food microbiology, vol. 77, no. 1-2, 25 July 2002, page 29 - page 38	8
A	EP 1020123 A1 (SITIA-YOMO S.P.A.), 19 July 2000 (19.07.2000), page 3, line 57; page 4, line 36, claim 2	1-14
A	WO 2005077391 A1 (SYNBIOTICS AB), 25 August 2005 (25.08.2005)	1-14
A	EP 0994183 A1 (RIKEN), 19 April 2000 (19.04.2000)	1-14
A	WO 02060276 A1 (VALIO LTD), 8 August 2002 (08.08.2002)	1-14

International patent classification (IPC)

A23L 1/03 (2006.01)
A61K 35/74 (2006.01)
A61P 31/16 (2006.01)
C12R 1/20 (2006.01)
C12R 1/225 (2006.01)
C12R 1/25 (2006.01)

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The password is **CGQHYLNVQD**.

Paper copies can be ordered at a cost of 50 SEK per copy from PRV InterPat (telephone number 08-782 28 85).

Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2006/001137

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 12-14
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 12-14 relate to a method of treatment of the human or animal body by therapy /Rule 39.1(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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