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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 August 2004 (12.08.2004)

PCT

(10) International Publication Number
WO 2004/067731 A1

(51) International Patent Classification[7]: C12N 1/20, A61K 45/00, A23L 1/03 // (C12N 1/20, C12R 1:00)

(21) International Application Number: PCT/SE2004/000098

(22) International Filing Date: 27 January 2004 (27.01.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0300245-8	31 January 2003 (31.01.2003)	SE
60/447,274	14 February 2003 (14.02.2003)	US

(71) Applicant *(for all designated States except US)*: **PROBI AB** [SE/SE]; Sölvegatan 41, S-223 70 Lund (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MOLIN, Göran** [SE/SE]; Examensvägen 2, S-224 67 Lund (SE). **AHRNÉ, Siv** [SE/SE]; Domarevägen 19, S-237 00 Bjärred (SE). **JEPPSSON, Bengt** [SE/SE]; Mätaregränden 8, S-226 47 Lund (SE).

(74) Agent: **AWAPATENT AB**; Box 5117, S-200 71 Malmö (SE).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NEW STRAINS OF BIFIDOBACTERIUM HAVING THE ABILITY TO PRODUCE GLUTAMINE

(57) Abstract: The invention refers to new strains of *Bifidobacterium*, especially of the species *Bifidobacterium infantis*, having the ability to survive in the intestinal tract and to produce glutamine and optionally arginine *in vivo*, as well as to compositions comprising said strains.

WO 2004/067731 A1

New strains of Bifidobacterium having the ability to produce glutamine.

Field of the invention

The present invention refers to new strains of *Bifidobacterum* having the ability to produce glutamine and optionally arginine *in vivo*.

Background of the invention

Glutamine is the most abundant amino acid in the body. It is a "nitrogen shuttle" between tissues and a fuel for enterocytes, colonocytes, lymphocytes and proliferating cells. The function of the gut is impaired in patients with glutamine deficiency, particularly because of the loss of protection against the translocation of bacteria and/or endotoxin from the gut lumen into the portal circulation. Glutamine depletion occurs in critically ill and injured patients, and may contribute to the high rate of infection and muscle depletion.

Enteral administration of glutamine has conferred health beneficial effects in patients with different indications. In intensive care unit patients with multiple organ dysfunction administration of glutamine decreased infectious complications in the patients (Houdijk *et al.*, Lancet, 352:772-776, 1998). Similar effects were observed in patients undergoing bone marrow transplantation receiving glutamine supplemented parenteral nutrition (Ziegler *et al.*, Annals Internal Medicine, 116:821-828, 1992). Another example is patients with short bowel syndrome, which had a substantial improvement of their absorption capacity following glutamine supplementation (Byrne *et al.*, Annals of Surgery, 222:243-255, 1995). Oral glutamine supplementation during and after chemotherapy has also been shown to signifi-

cantly reduce both the duration and the severity of chemotherapy-associated stomatitis. It was concluded that oral glutamine appeared to be a simple and useful measure to increase the comfort of many patients at high risk of developing mouth sores as a consequence of intensive cancer chemotherapy (Anderson *et al.*, *Cancer* 1998; 83:1433-9). Nutritional supplementation of glutamine after intensive exercise has also decreased the incidence of infections, particularly of upper respiratory tract infections (Castell, *Amino acids* 2001; 20(1):49-61). However, the precise effect of glutamine on immunodepression has not yet been established.

A major technical difficulty with glutamine is that during processing and storage glutamine is easily converted to glutamic acid (glutamate), i.e. glutamine is a relatively unstable compound that is difficult to incorporate into formulas intended for oral administration. Furthermore, the orally administered glutamine will in the sour environment of the stomach to a high degree be converted into glutamic acid and never reach the intestine and be absorbed as glutamine.

Arginine enhances the immune function and promotes wound healing. Administration of arginine has been used in postoperative patients and patients under intensive care. In most clinical studies arginine has been administered together with other substances such as RNA and fish oil. There are indications that administration of arginine modulates post-operative immune response. Daly, John E., et al., *Surgery* 112:55-67, 1992 show that enteral nutrition with supplemental arginine, RNA and omega-3-fatty acids in patients after operation improves the immune defence through different mechanisms. Arginine reduces complications in patients undergoing chemoradia-

tion and surgery (Tepaske *et al.*, 2001; *Lancet* 358: 696-701), and reduces length of stay for intensive care unit patients (Bauer *et al.*, 1995, *Critical Case Medicine* 23: 436-449).

An increased survival could be observed in animals fed with an arginine-supplemented diet. Quantitative colony counts and the calculated percentage of remaining viable bacteria showed that the ability to kill translocated organisms was signify-cantly enhanced in animals receiving arginine (Adawi, D., *et al.*, 1997, Hepatology 25: 642-647).

Strains of *Bifidobacterium* spp., that is bifidobacteria, are often present in high numbers in the human colon, especially in breast fed babies. Bacteria from the *Bifidobacterium* spp. are regarded as probiotics, that is live bacteria that upon ingestion provide health beneficial effects to the host. High numbers of *Bifidobacterium* spp. in colon have been claimed to have health beneficial effects. However, the course of action of said beneficial effects is largely unknown.

Prior art

Matteuzzi *et al.* [*Ann. Microbiol. (Inst. Pasteur)* 1978;129 B, 175-181] investigated a large number of bifido-bacteria from different habitats for their capacity to release free amino acids in culture broths. The data obtained indicated that several species of bifidobacteria were capable of synthesising all the amino acid needed for growth and also to liberate said compounds into the culture broths. *B. thermophilum*, *B. bifidum* and *B. adolescentis* were found to be the best producers, and the amino acids most commonly found in the culture broths were mainly alanine, valine, and aspartic acid. It was

speculated that *Bifidobacterium* spp. play a role in the amino acid metabolism of the gastrointestinal tract.

WO 01/83700 (University of Maryland) discloses a composition and method for treating and preventing gastro-intestinal injury, neonatal necrotising enterocolitis (NEC) and bacterial sepsis. The composition includes a combination of Gram (+) bacteria, in particular *Lactobacillus* and *Bifidobacterium*, and glutamine, and should be orally or naso-orally administrated. The composition is said to block translocation of bacterial agents such as Gram (-) bacteria.

Glutamine administered intravenously is very efficient but expensive and complicated. A better way to administer glutamine would of course be to use a bacterial strain having the ability to produce substantial amounts of glutamine in the intestines. Until now, however, no such strains have been described.

Summary of the invention

It has now surprisingly been found that specific strains of *Bifidobacterium* are capable of producing glutamine in a growth medium mimicking the environment in the human colon. Said strains can thus be used for producing glutamine *in vivo* after oral or enteral administration to a mammal, especially a human. Some of the strains are also capable of producing arginine.

Brief description of the drawings

Figure 1 is a schematic representation of the electrophoretic patterns obtained with Restriction Fragment Length Polymorphism (RFLP) for the strains CURE 19, CURE 21, CURE 26, CURE 28, and CURE 29, obtained by cleaving chromosomal DNA with EcoRI and Hind III, respectively, followed by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering)

of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation. Dig-labelled Molecular weight DNA marker II (Roche) and Molecular weight DNA marker VI (Roche) were used as standard.

Figure 2 shows a photograph of separated DNA fragments obtained by cleaving chromosomal DNA of strains CURE 19 (lane 1), CURE 29 (lane 2) and CURE 28 (lane 3) with the restriction enzyme EcoRI (Restriction Endonuclease Analysis, REA). High Molecular Weight DNA marker (BRL) and DNA molecular weight marker VI (Roche) were used as standard (lane 4).

Figure 3 shows a photograph of separated DNA fragments obtained by cleaving chromosomal DNA of strains CURE 19 (lane 2), CURE 28 (lane 3) and CURE 29 (lane 4) with the restriction enzyme Hind III. High Molecular Weight DNA marker (BRL) and DNA molecular weight marker VI (Roche) were used as standards (lanes 1 and 5).

Figure 4 shows a photograph of separated DNA fragments obtained by cleaving chromosomal DNA of strains CURE 21 (lane 1) and CURE 26 (lane 2) with the restriction enzyme EcoRI. High Molecular Weight DNA marker (MD, USA) and DNA molecular weight marker VI (Roche) were used as standard (lane 3).

Figure 5 shows a photograph of separated DNA fragments obtained by cleaving chromosomal DNA of strains CURE 21 (lane 2) and CURE 26 (lane 3) with the restriction enzyme Hind III . High Molecular Weight DNA marker (MD, USA) and DNA molecular weight marker VI (Roche) were used as standards (lanes 1 and 4).

Figure 6. Disease activity index on day 4, 5, 6 and 7. * denotes $p<0.05$ compared to colitis control, ¤ denotes $p<0.05$ compared to *L. gasseri*, # denotes $p<0.05$ compared to *L. paracasie*.

Description of the invention

The invention refers to a strain of *Bifidobacterium* having the ability to survive in the intestinal tract and to produce glutamine *in vivo*. Especially glutamine is produced in the human colon. Survive in this context means that the bacterial strains have been isolated on different sampling occasions in faeces from an individual. Thus, the bacteria obviously grow and survive in their host for a while. The bifidobacterial strains of the invention can grow on nutrient media having a pH below 7, especially 5.5-6.5. Rogosa agar is one example of such a medium, another is MRS.

According to a preferred aspect the new strains also have the ability to assimilate ammonia.

The invention especially refers to strains belonging to the species *Bifidobacterium infantis*.

According to a specific aspect the invention also refers to a strain of *Bifidobacterium* which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 2840 kb, obtained by cleaving chromosomal DNA with Hind III, followed by separation of fragments by agarose gel electrophoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation. Such strains are, for example, the strains, deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on August 23, 2002, *Bifidobacterium infantis* CURE 19 (DSM 15158); *Bifidobacterium infantis* CURE 21 (DSM 15159); *Bifidobacterium infantis* CURE 26 (DSM 15160); *Bifidobacterium infantis* CURE 28 (DSM 15161); *Bifidobacterium infantis* CURE 29 (DSM 15162).

According to another aspect the invention also refers to a strain of *Bifidobacterium* which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 895 kb, obtained by cleaving chromosomal DNA with EcoRI, followed by separation of fragments by agarose gel electro-phoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation. According to a preferred aspect said strain is able to produce glutamine without reducing glutamic acid.

The invention especially refers to the strains *Bifidobacterium infantis* CURE 21 and *Bifidobacterium infantis* CURE 26, which were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on August 23, 2002 and were given the accession numbers DSM 15159 and DSM 15160, respectively, or variants thereof. Said strains are able to produce glutamine without reducing glutamic acid.

According to another aspect the invention also refers to a strain of *Bifidobacterium* which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 3420 kb, obtained by cleaving chromosomal DNA with EcoRI, followed by separation of fragments by agarose gel electro-phoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation. According to a preferred aspect said strain has the ability to produce arginine.

The invention also especially refers to the following strains, which have all been deposited at the Deut-

sche Sammlung von Mikroorganismen und Zellkulturen GmbH on August 23, 2002, and been given a deposition number, that is *Bifidobacterium infantis* CURE 19, DSM 15158, *Bifidobacterium infantis* CURE 28, DSM 15161, and *Bifidobacterium infantis* CURE 29, DSM 15162, and to variants thereof. Said strains are able to produce in addition to glutamine also arginine.

The new strains have all been isolated from faeces from young children and selected by culturing on agar at a pH below 7. The strains have subsequently been characterised by ribotyping and REA.

Another object of the invention is a composition comprising one or more strains of *Bifidobacterium* of the invention in combination with a carrier. Examples of carriers are oatmeal gruel, lactic acid fermented foods, inulin, lactulose, fructo-oligosaccharides, resistent starch, β-glucans and guar gum. In order to improve the proliferation of the bifidobacteria and increase the production of glutamin and arginine, respectively, and enhance the ammonia assimilation in colon dietary fibres should be added to the composition. Dietary fibres are for instance inulin, fructo-oligo-saccharides, maltodextrins, β-glucans and guar gum. The invention thus also refers to a composition as described comprising in addition dietary fibres.

The compositions of the invention, such as suspensions, tablets, capsulas, powders, can be administrated orally. They can also be administrated as an enema.

The composition of the invention can be a food composition wherein the carrier is a food product. The glutamine producing *Bifidobacterium*-strains can be given to young children, elderly people, athletics and ordinary consumers that wish to keep-fit to improve muscle func-

tion and avoid immune depression following exercise. The arginine producing *Bifidobacterium*-strains can be given to ordinary consumers that want to keep-fit and avoid negative influence on immune function.

The composition of the invention can be a pharmaceutical composition, wherein the carrier is a therapeutically acceptable carrier. The glutamine producing strains, as well as the arginine producing strains can in addition be used in formulas for enteral feeding.

The ammonia assimilating *Bifidobacterium* strains of the invention can be given to patients with temporary kidney failure such as seen in patients under intensive care following surgery and complications or following other diseases, such as severe infections, intoxications. In such cases the kidney function can be expected to return and a treatment aimed at reducing the nitrogen load from the gut may avoid the need for dialysis. The ammonia assimilating strains can be administered to patients with liver failure and encephalopathy, e.g. in hepatitis or intoxication or following alcohol abuse. A reduced absorption of nitrogenous substances from the gut will in those situations improve encephalopathy and liver function. The ammonia assimilating *Bifidobacterium* strains can be given to young children, elderly people or consumers with underlying diseases that hamper their liver capacity for the conversion of ammonia to urea or having an increased absorption of nitrogen from the gut, e.g. patients with chronic renal or liver failure of small to moderate degree not yet in need of transplantation or dialysis.

According to a special aspect the composition of the invention can also comprise one or more *Lactobacillus* strains.

In addition to the optional beneficial effects of the lactobacilli per se, said bacteria can protect the bifidobacteria from the harmful influence of oxygen.

According to another aspect, the invention refers to one or more strains belonging to the species *Bifidobacterium infantis* for use in therapy.

The invention refers to the use of one or more of the strains *Bifidobacterium infantis* CURE 19, DSM 15158; *Bifidobacterium infantis* CURE 21, DSM 15159; *Bifidobacterium infantis* CURE 26, DSM 15160; *Bifidobacterium infantis* CURE 28, DSM 15161; *Bifidobacterium infantis* CURE 29, DSM 15162; or a variant thereof for the preparation of a medicament for treatment of intensive care patients with multiple organ dysfunction and intestinal failure, for prophylaxis in chemotherapy patients and patients with inflammatory diseases and postoperative administration after major surgery.

Experimental

Isolation of Strains

All strains were isolated from faeces of young children, age one week to one year. The faeces samples were serial diluted in a dilution solution (0.9% [w/v] NaCl, 0.1% [w/v] peptone, 0.1% [w/v] Tween 80, 0.02% [w/v] cystein-HCl) and spread on Rogosa agar plates. Isolates were selected with respect to their ability to grow on Rogosa agar, pH 5.4, and repeated isolation from one individual. The isolates were picked from the Rogosa agar plates after incubation at 37°C for 72 hours. They were identified to genus level by genus specific PCR (Roy *et al.*, 2000; *FEMS Microbiological Letters* 191:17-24) and to species level by 16S rDNA sequencing (Pettersson *et al.*, 2002; *Systematic and Applied Microbiology* 23:332-336). The strains could be isolated at least twice from

the same individual with one to four weeks between the samplings, which strongly indicate that the strains had a certain ability to colonise the GI-tract. The strains were identified by ribotyping, that is Restriction Fragment Length Polymorphism, RFLP, of the 16S rRNA gene, and by REA, that is Restriction Endonuclease Analysis.

The following strains were isolated:

1) *Bifidobacterium infantis* CURE 19; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "sour" smell after fermentation.
2) *Bifidobacterium* CURE 20; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "sour" smell after fermentation.
3) *Bifidobacterium infantis* CURE 21; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "sour" smell after fermentation.
4) *Bifidobacterium* CURE 22; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "nice" smell after fermentation.
5) *Bifidobacterium* CURE 23; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "nice" smell after fermentation.
6) *Bifidobacterium infantis* CURE 24; able to grow on Rogosa agar and to ferment oatmeal gruel to some degree, "nice" smell after fermentation.
7) *Bifidobacterium* CURE 25; able to grow on Rogosa agar and to ferment oatmeal gruel.
8) *Bifidobacterium infantis* CURE 26; able to grow on Rogosa agar and to ferment oatmeal gruel.
9) *Bifidobacterium dentium* CURE 27; able to grow on Rogosa agar and to ferment oatmeal gruel.
10) *Bifidobacterium infantis* CURE 28; able to grow on Rogosa agar and to ferment oatmeal gruel.

11) *Bifidobacterium infantis* CURE 29; able to grow on Rogosa agar and to ferment oatmeal gruel.

12) *Bifidobacterium infantis* CURE 30; able to grow on Rogosa agar and to ferment oatmeal gruel.

Glutamine production

The isolated strains 1-12 were tested for production of glutamine in broth by the following procedure.

The test strains were cultured at 37°C for 4 days in a growth medium (broth) modified from the medium described by Matteuzzi *et al.* (Ann. Microbil. (Inst. Pasteur, 1978, 129 B: 175-181). The broth was composed of: sodium acetate, 10 g/l; ascorbic acid, 10 g/l; ammonium sulphate ($(NH_4)_2SO_4$), 5 g/l; dipotassium hydrogen phosphate (K_2HPO_4), 3 g/l; potassium di-hydrogen phosphate (KH_2PO_4), 3 g/l; $MgSO_4$ x $7H_2O$, 0.32 g/l; $FeSO_4$ x $7H_2O$, 0.01 g/l; $MnSO_4H_2O$, 0.007 g/l; NaCl, 0.01 g/l; yeast extract, 0.5 g/l; glucose, 20 g/l; Tween 80, 1 ml/l. pH was adjusted with 1 M NaOH to 6.18-6.24 prior to autoclavation.

The glutamine concentration in the broth was measured before inoculation with bacteria and after growth of the test strain. After growth, the culture was centrifuged and sterile filtered, and the cell-free supernatant subsequently frozen at -80°C. The amino acids were analysed in an automated analyser (Biochrom 20, Pharmacia Biotech) after addition of sulpho-salicylic acid and pH adjustment with lithiumhydroxide.

The increase in the glutamine and glutamic acid concentration in the broth after the growth of different test strains is shown in Table 1 below.

Table 1. Increased concentration of glutamine and reduction/production of glutamic acid in the culture broth after growth of the tested *Bifidobacterium* strains.

Test strain	Glutamine (μmol/l)	Glutamic acid [reduction (R), production (P) in μmol/l]
B. infantis CURE 19	53 (29-67)[#]	R
Bifidobacterium CURE 20	4.2 (3.5-4.8)[§]	R
B. infantis CURE 21	37 (36-38)[#]	P 37 (31-41)
Bifidobacterium CURE 22	16 (14-18)[§]	R
Bifidobacterium CURE 23	8.9 (8.1-9.6)[§]	R
B. infantis CURE 24	3.0 (0-5.9)[§]	R
B. infantis CURE 25	35	R
B. infantis CURE 26	36	P 60
B. dentium CURE 27	7.4	R
B. infantis CURE 28	35	R
B. infantis CURE 29	28	R
B. infantis CURE 30	37	R

[#] The mean value of three samples derived from three separate cultures

[§] The mean value of two samples derived from two separate cultures

All test strains produced some glutamine but only seven of the 12 tested strains had a relatively high production of glutamine (>20 μmol/l). For all but two strains (CURE 21 and CURE 26), the increase in glutamine occurred along with a strong reduction of glutamic acid in the medium. Medium to which no bacteria were added had an average start concentration of glutamic acid of 223 μmol/l, while the content of glutamine was zero. The three strains CURE 20, CURE 23 and CURE 24 consumed all

available glutamic acid in the medium while CURE 28 decreased the concentration of glutamic acid with 247 μmol/l, CURE 29 with 220 μmol/l, CURE 22 with 187 μmol/l, CURE 19 with 128 μmol/l, CURE 30 with 160 μmol/l, CURE 27 with 151 μmol/l, and CURE 25 with 47 μmol/l. It can be speculated if these bacteria convert glutamic acid to glutamine. Two strains, CURE 21 and CURE 26, produced glutamine without any reduction of the glutamic acid concentration in the medium. In contrast to the other strains, they increased the level of glutamic acid in the broth.

Arginine production

The amino acid production of each test strain was measured after growth of the test strain at 37°C for 4 days in a growth medium (broth) modified from the medium described by Matteuzzi *et al.* (1978). The broth was composed of: sodium acetate, 10 g/l; ascorbic acid, 10 g/l; ammonium sulphate ($(NH_4)_2SO_4$), 5 g/l; dipotassium hydrogen phosphate (K_2HPO_4), 3 g/l; potassium dihydrogen phosphate (KH_2PO_4), 3 g/l; $MgSO_4$ x $7H_2O$, 0.32 g/l; $FeSO_4$ x $7H_2O$, 0.01 g/l; $MnSO_4H_2O$, 0.007 g/l; NaCl, 0.01 g/l; yeast extract, 0.5 g/l; glucose, 20 g/l; Tween 80, 1 ml/l. pH was adjusted with 1 M NaOH to 6.18-6.24 prior to autoclavation.

The amino acid concentration in the broth was measured before inoculation with bacteria and after growth of the test strain. After growth, the culture was centrifuged and sterile filtered, and the cell-free supernatant subsequently frozen at -80°C. The amino acids were analysed in an automated analyser (Biochrom 20, Pharmacia Biotech) after addition of sulphosalicylic acid and pH adjustment with lithium-hydroxide.

The increase in the concentration of arginine and citrulline in the broth after the growth of the different test strains is shown in Table 2.

Table 2. Increased concentration of arginine and citrulline in the broth after growth of the test strains.

Test strain	Arginine (μmol/l)	Citrulline (μmol/l)
B. infantis CURE 19	11 (8.8-14)[#]	52(9.2-136)[#]
Bifidobacterium CURE 20	0[§]	4.1 (4.4-3.7)[§]
B. infantis CURE 21	0[#]	0[#]
Bifidobacterium CURE 22	0[§]	6.3 (5.8-6.7)[§]
Bifidobacterium CURE 23	2.0 (0-3.9)[§]	6.1 (5.4-6.8)
B. infantis CURE 24	0[§]	0[§]
B. infantis CURE 25	0	4.2
B. infantis CURE 26	2.6	0
B. dentium CURE 27	4.9	0.0
B. infantis CURE 28	37	202
B. infantis CURE 29	26	13
B. infantis CURE 30	2.0	5.7

[#] The mean value of three samples derived from three separate cultures

[§] The mean value of two samples derived from two separate cultures

Three of the 12 test strains increased the concentration of arginine with more than 10 μmol/l, that is CURE 19 CURE 28 and CURE 29. Two of said strains also had a strikingly strong production of citrulline (>100 μmol/l), that is CURE 19 and CURE 28. Citrulline is produced either by deamination of arginine or through NO-synthase and generation of NO. The citrulline production also reflects arginine production in an earlier step.

Also other amino acids penetrated out into the broth during growth of the test strains, see Table 3. CURE 21 did not produce any citrulline or arginine but significant amounts of asparagine acid and tyrosine. CURE 26 did not produce any citrulline but very small amounts of arginine and a wide spectrum of different amino acids. Furthermore, CURE 26 was the only test strain that increased the concentration of proline in the broth. All test strains produced threonine.

TABLE 3. Increase in amino acid concentrations (μmol/l), in addition to glutamine, glutamic acid, arginin, and citrullin in the broth after growth of the test strains.

Strain	Thr	Tyr	Cys	Asp	Ala	Gly	Ile
CURE 19	39	15	0	23	15	0	0
CURE 21	51	21	0	96	0	0	0
CURE 26	46	46	12	151	61	0	6
CURE 28	44	12	14	0	0	0	0
CURE 29	35	28	0	0	2	0	0

Thr-threonine; Tyr-tyrosine; Cys-cysteine; Asp-asparagine acid; Ala-alanine; Gly-glycine; Ile-isoleucine

The values represent mean values of three or two samples derived from three or two separate cultures.

Genotypic identification

REA

The strains were examined as to the cleavage pattern of the chromosomal DNA, through restriction-endonuclease analysis - REA - method according to Ståhl M, Molin G, Persson A, Ahrné S & Ståhl S, International Journal of Systematic Bacteriology, 40:189-193, 1990, and further developed by Johansson, M-L, et al., International Journal of Systematic Bacteriology 45:670-675, 1995. Schematically REA can be described as follows: Chromo-

somal DNA from the strains involved in the study were prepared and cleaved by restriction endonucleases. 0.75 µg of each DNA was separately digested at 37°C for 4 h with 10 units of EcoRI and Hind III; each endonuclease was used separately. The cleaved DNA fragments are separated as to size by gel electrophoresis using submerged horizontal agarose slab gels. The gels consisted of 150 ml of 0.9 % agarose (ultrapure DNA grade; low electro-endo osmosis; BioRad Laboratories, Richmond, USA) and were cast as slab gels (150 by 235 mm). 0.2 µg of the High Molecular Weight DNA marker (Bethesda Research Laboratories, MD, USA) together with 0.5 µg of a DNA molecular weight marker VI (Roche, Germany) were used as standards. Minimal band distortion and maximal sharpness were achieved by applying the sample DNA in Ficoll loading buffer (2g of Ficoll, 8 ml of water, 0.25% bromphenol).

Gels were run at a constant voltage of 40V for 18h at about 6-8°C. The buffer (89 mM Tris, 23 mM H_3PO_4, 2 mM sodium EDTA, pH 8.3) was recirculated during the running period. Thereafter, the gels were stained for 20 minutes in ethidium bromide (2 µg/ml) and destained in distilled water, visualized at 302 nm with a UV transilluminator (UVP Inc., San Gabriel, USA) and photographed. This way of running the gel electrophoresis gave well distributed and relatively well-separated band down to a molecular weight of 1.2×10^6.

RFLP *of the 16S rRNA gene (ribotyping)*

Preparation of chromosomal and Restriction Endonuclease Analysis of chromosomal DNA was performed as described earlier (Ståhl *et al.*, 1990, Ståhl *et al.*, 1994).

The probe was a 420 bp fragment (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei*

ssp. *pseudoplantarum* DSM 20008, obtained by PCR and labelled by DIG DNA labelling technique according to the instructions supplied by the manufacturer (Boehringer Mannheim, Bromma, Sweden). The amount of probe used in the reactions was 50 ng.

Southern blot hybridisation. One µg of the chromosomal DNA was digested with 10U of *Eco*RI and *Hind*III (Boehringer Mannheim) for 4 h at 37°C. Separation of restriction fragments by agarose gel electrophoresis was performed according to Ståhl *et al.*, 1994. DIG-labelled Molecular weight DNA marker II and Molecular weight DNA marker VI were used as standards (Roche, Germany). The DNA was immobilised on a positively charged nylon membrane (Roche) by baking for 30 min at 120°C. Prehybridisation, hybridisation and chemoluminescent detection with the substrate CSPD® (Roche) were performed according to the instructions supplied by the manufacturer. The hybridisation temperature was 68°C. The test resulted in one single band with a molecular weight of about 2840 kb in for all strains (CURE 19, 21, 26, 28 and 29) when chromosomal DNA was cleaved by Hind III. When chromosomal DNA was cleaved by EcoRI, the genes for 16S rRNA ended up on a single fragment with the molecular weight of about 895 kb in strains CURE 21 and 26, while another single band with a molecular weight of about 3420 kb was obtained for strains CURE 19, 28 and 29.

Test in vivo

Test 1. Effects of *Lactobacillus* and *Bifidobacterium* strains on DDS induced colitis in rat

The aim of this study was to compare the effects of *Lactobacillus* and *Bifidobacterium* strains on DDS (Dextran Sodium Sulfate) induced colitis in rat.

Sprague Dawley rats were divided into six groups, one control group (colitis without administration of bacteria), and five groups to which different bacterial strains (*Lactobacillus plantarum* 299v, *Lactobacillus paracasei* 8700:2, *Lactobacillus gasseri* LG1, *Bifidobacterium* 3B1 and *Bifidobacterium infantis* CURE 19, respectively) were administered. The bacterial strains were administered orally for 7 days prior to the induction of colitis (day 0) and were continuously administered for 7 days in combination with DDS (5% w/v dissolved in water). The degree of colitis were determined daily with DAI (Disease Activity Index). Sampling was performed day 14 and bacterial translocation and quantity in the intestine determined.

In the groups receiving *Lactobacillus plantarum* 299v, *Bifidobacterium* 3B1 and *Bifidobacterium* CURE 19, DAI decreased significantly day 4, 5, 6 and 7 compared to the control group. In addition, DAI was significantly lower in the group receiving *B. infantis* CURE 19 compared to the groups receiving *Lactobacillus paracasei* 8700:2 and *Lactobacillus gasseri* LG1 after 6 and 7 days. Compared to the control colitis, the bacterial translocation to the mesenteric lymph nodes decreased significantly in all groups as well as the translocation of *Enterobacteriaceae* to the liver.

In conclusion, oral administered bacterial strains of *Lactobacillus plantarum* 299v, *Lactobacillus paracasei* 8700:2, *Lactobacillus gasseri* LG1, *Bifidobacterium* 3B1 and *Bifidobacterium infantis* CURE 19 are able to exercise positive effects by decreasing translocation on experimental colitis in rats. *Lactobacillus plantarum 299v*, *Bifidobacterium* 3B1 and *Bifidobacterium infantis* CURE 19 showed the most evident effects in improved DAI in the

DDS induced colitis rat. *Bifidobacterium infantis* CURE 19 was somewhat more effective in counteracting the colitis than the others after 6d and 7d of treatment. Please see figure 6.

Test 2. Survival of *Lactobacillus* and/or *Bifidobacterium* in the GI-tract after oral administration

Oral administration of *Lactobacillus* or *Bifidobacterium* provides different positive effects, e.g. preventing antibiotic associated diarrhoea (D'Souza et al., 2002, BMJ 324:1361)

Positive effects may be achieved provided that the bacteria survive the oral administration and have the ability to survive and thrive in the intestines. To find suitable strains that can be used as probiotics, a number of possible candidate strains are administered orally to healthy subjects. Determination of the frequency of the administered strains in the faeces identifies strains with high survival ability. The aim of this study is to find orally administrable bacterial strains with a superior ability to survive and thrive in the human GI-tract.

Design of the study

For four weeks fourteen voluntary subjects are to drink a mixture of approximately 20 different strains of *Lactobacillus* and *Bifidobacterium*. All strains are representatives of strains occurring in humans or in lactic acid producing food. Faecal sampling will be taken before administration, after 3 weeks of administration and one week post administration. The bacterial flora is determined by viable count and RAPD (Randomly Amplified Polymorphic DNA; Johansson *et al.*, 1995).

Conclusion

The results show that the selected strains, that is *Bifidobacterium infantis* CURE 19, DSM 15158; *Bifidobacterium* CURE 21, DSM 15159; *Bifidobacterium infantis* CURE 26, DSM 15160; *Bifidobacterium infantis* CURE 28, DSM 15161; and *Bifidobacterium infantis* CURE 29, DSM 15162, can survive the passage through the gastrointestinal tract after oral administration. Studies in rats also show a potential of exercising positive effects on experimental colitis.

CLAIMS

1. A strain of *Bifidobacterium* having the ability to survive in the intestinal tract and to produce glutamine *in vivo*.

2. A strain according to claim 1, having the ability to assimilate ammonia.

3. A strain according to claims 1 or 2, which strain belongs to the species *Bifidobacterium infantis*.

4. A strain according to any of claims 1-3, which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 2840 kb obtained by cleaving chromosomal DNA with Hind III, followed by separation of fragments by agarose gel electrophoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation.

5. A strain according to any of claims 1-4, which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 895 kb, obtained by cleaving chromosomal DNA with EcoRI, followed by separation of fragments by agarose gel electrophoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (position 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation.

6. A strain according to any of claims 1-4, which has the 16S rRNA genes located on single DNA fragments with molecular sizes of about 3420 kb, obtained by cleaving chromosomal DNA with EcoRI, followed by separation of fragments by agarose gel electrophoresis, and by hybridisation with a DIG-labelled 420 bp fragment probe (po-

sition 506 to 926, *E. coli* numbering) of the 16S rRNA gene of *L. casei* subsp. *pseudoplantarum* DSM 20008 using Southern blot hybridisation.

7. A strain according to any of claims 1-5, which is the strain *Bifidobacterium infantis* CURE 21, DSM 15159, or a variant thereof having essentially the same REA-pattern.

8. A strain according to any of claims 1-5, which is the strain *Bifidobacterium infantis* CURE 26, DSM 15160, or a variant thereof having essentially the same REA-pattern.

9. A strain according to any of claims 1-4 and 6, having the ability to produce arginine.

10. A strain according to any of claims 1-4, 6 and 9, which is the strain *Bifidobacterium infantis* CURE 19, DSM 15158, or a variant thereof having essentially the same REA-pattern.

11. A strain according to any of claims 1-4, 6 and 9, which is the strain *Bifidobacterium infantis* CURE 28, DSM 15161, or a variant thereof having essentially the same REA-pattern.

12. A strain according to any of claims 1-4, 6 and 9, which is the strain *Bifidobacterium infantis* CURE 29, DSM 15162, or a variant thereof having essentially the same REA-pattern.

13. A composition comprising one or more strains of *Bifidobacterium* according to any of claims 1 to 12 in combination with a carrier.

14. A composition according to claim 13, characterised in that the carrier is oatmeal gruel.

15. A composition according to claim 13 or 14, characterised in also comprising dietary fibres.

16. A composition according to any of claims 13-15, characterised in being a food composition.

17. A composition according to any of claims 13-15, characterised in being a pharmaceutical composition wherein the carrier is a therapeutically acceptable carrier.

18. A composition according to any of claims 13-17, characterised in also comprising one or more *Lactobacillus* strains.

19. One or more strains belonging to the species *Bifidobacterium infantis* for use in therapy.

20. Use of one or more of the strains *Bifidobacterium infantis* CURE 19, DSM 15158; *Bifidobacterium infantis* CURE 21, DSM 15159; *Bifidobacterium infantis* CURE 26, DSM 15160; *Bifidobacterium infantis* CURE 28, DSM 15161; *Bifidobacterium infantis* CURE 29, DSM 15162; or a variant thereof for the preparation of a medicament for treatment of intensive care patients with multiple organ dysfunction and intestinal failure, for prophylaxis in chemotherapy patients and patients with inflammatory diseases, or for postoperative administration after major surgery.

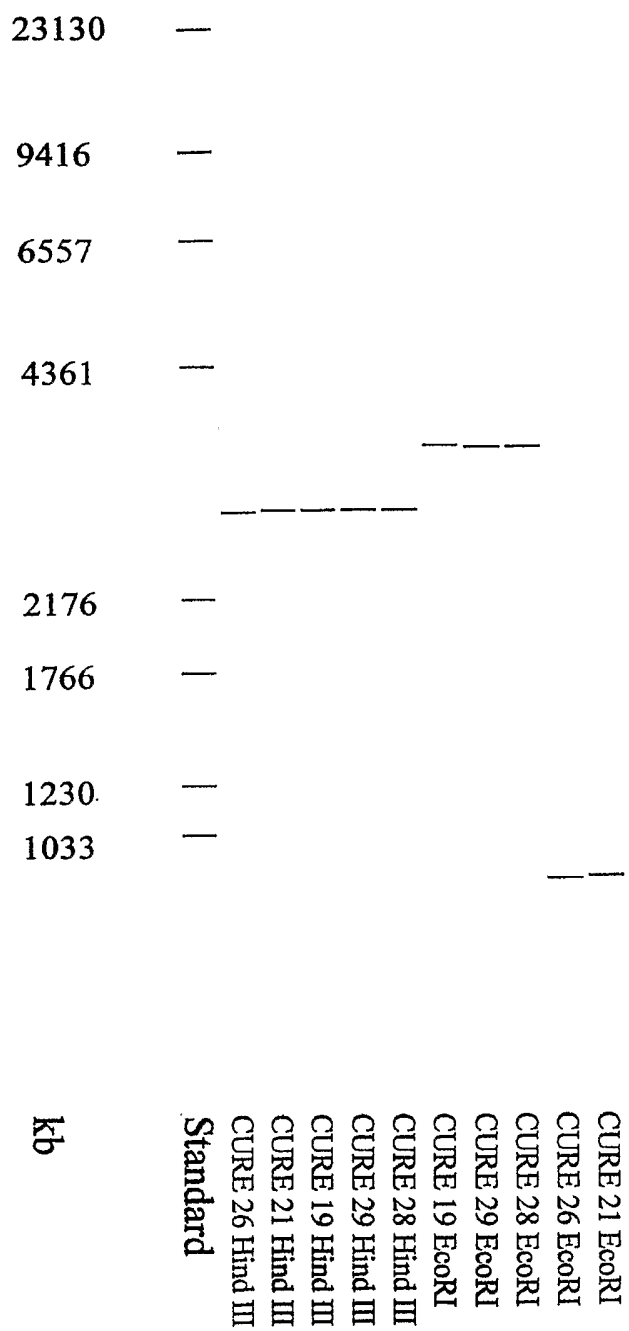
23130
9416
6557
4361
2176
1766
1230
1033
kb
Standard
CURE 26 Hind III
CURE 21 Hind III
CURE 19 Hind III
CURE 29 Hind III
CURE 28 Hind III
CURE 19 EcoRI
CURE 29 EcoRI
CURE 28 EcoRI
CURE 26 EcoRI
CURE 21 EcoRI

FIGURE 1

FIGURE 2

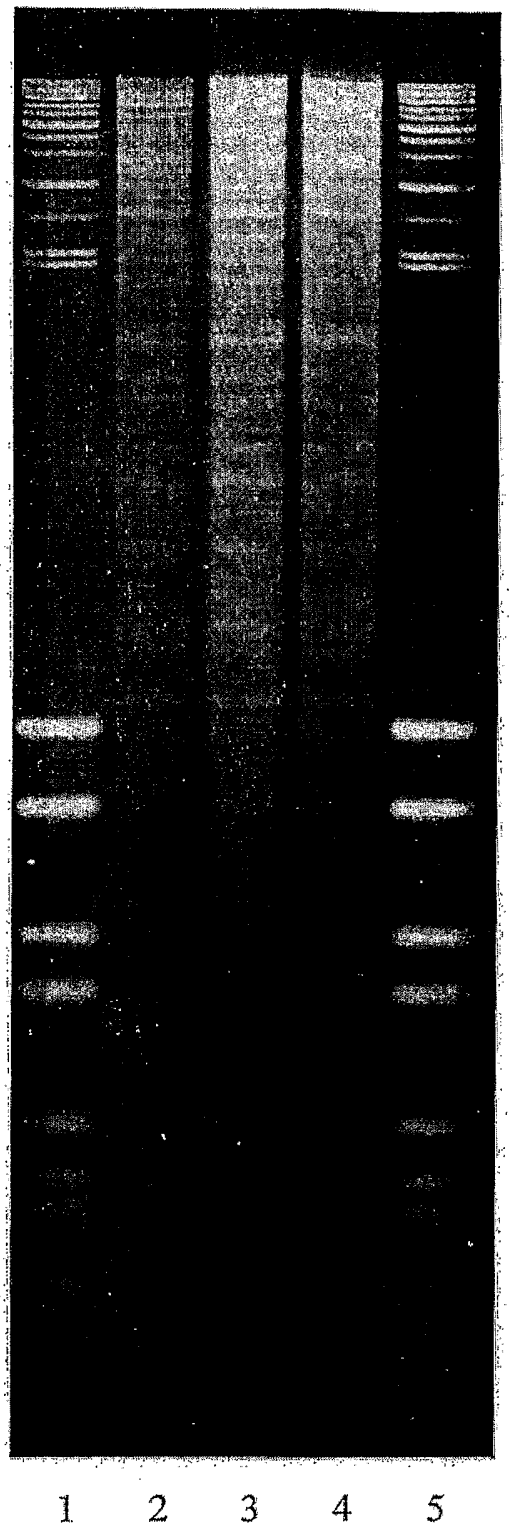

FIGURE 3

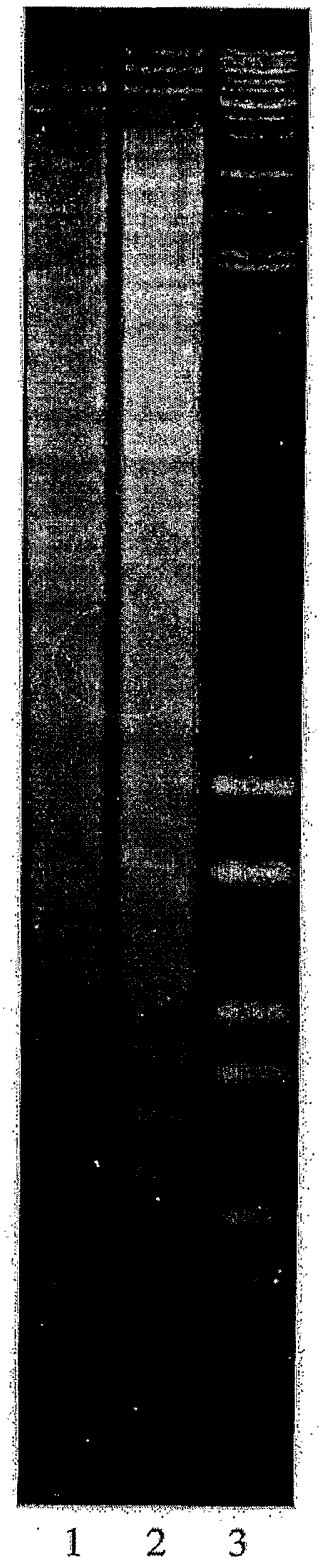

FIGURE 4

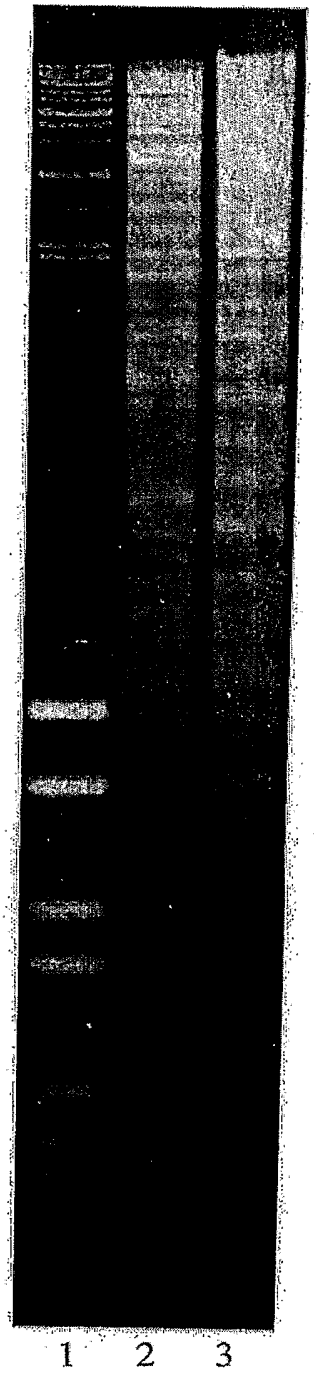

FIGURE 5

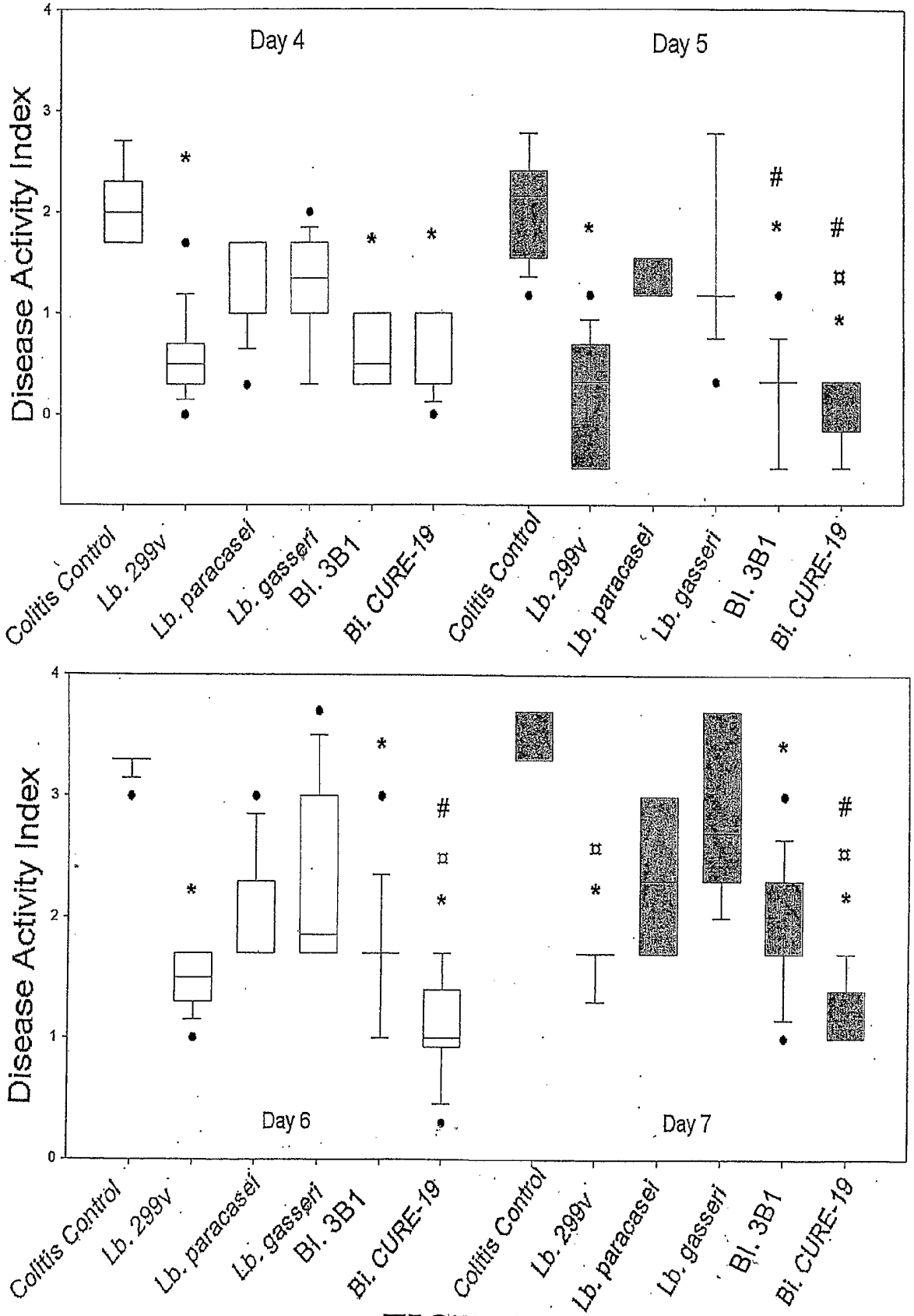
Day 4
Day 5
Disease Activity Index
0
1
2
3
4
Colitis Control
Lb. 299v
Lb. paracasei
Lb. gasseri
Bl. 3B1
Bi. CURE-19
Colitis Control
Lb. 299v
Lb. paracasei
Lb. gasseri
Bl. 3B1
Bi. CURE-19
Day 6
Day 7
Disease Activity Index
0
1
2
3
4
Colitis Control
Lb. 299v
Lb. paracasei
Lb. gasseri
Bl. 3B1
Bi. CURE-19
Colitis Control
Lb. 299v
Lb. paracasei
Lb. gasseri
Bl. 3B1
Bi. CURE-19

FIGURE 6

PCT/SE2004/000098

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Cure 19	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15158

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(x) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-08-23 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b, D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wätzs Date: 2002-08-30

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Probi AB Address: Ideon Sölvegatan 41 223 70 LUND Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15158 Date of the deposit or the transfer[1]: 2002-08-23

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-08-26 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Wetz Date: 2002-08-30

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

PCT/SE2004/000098

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
CURE 21	DSM 15159

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-08-23 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH
Address:	Mascheroder Weg 1b D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

U. Weiss

Date: 2002-08-30

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Probi AB Address: Ideon Sölvegatan 41 223 70 LUND Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15159 Date of the deposit or the transfer[1]: 2002-08-23

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-08-26 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiss Date: 2002-08-30

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CURE 26	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15160

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-08-23 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wells Date: 2002-08-30

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Probi AB Address: Ideon Sölvegatan 41 223 70 LUND Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15160 Date of the deposit or the transfer[1]: 2002-08-23

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-08-26 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH
Address:	Mascheroder Weg 1b D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

V. Weiks

Date: 2002-08-30

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CURE 28	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15161

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(x) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-08-23 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b, D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): C. Weiss Date: 2002-08-30

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Probi AB Address: Ideon Sölvegatan 41 223 70 LUND Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15161 Date of the deposit or the transfer[1]: 2002-08-23

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-08-26 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiss Date: 2002-08-30

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CURE 29	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15162

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-08-23 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wirths Date: 2002-08-30

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon
Sölvegatan 41
223 70 LUND
Sweden

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Probi AB Address: Ideon Sölvegatan 41 223 70 LUND Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15162 Date of the deposit or the transfer[1]: 2002-08-23

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-08-26 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wehs Date: 2002-08-30

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/000098

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 1/20, A61K 45/00, A23L 1/03 // (C12N 1/20, C12R 1/00)
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)

IPC7: C12N

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, PAJ, BIOSIS, MEDLINE, CHEM. ABS DATA, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN International, File CAPLUS, CAPLUS accession no. 1991:554686, Document no. 115:154686, Yano, Toshihiro et al: "Selective growth and mechanism of related enzymes of bifidobacteria"; & Rakuno Kagaku, Shokuhin no Kenkyu (1991), 39(6), A229-A237, abstract	1-12
Y	--	13-18,20
Y	Cancer, Vol. 83, 1998, Peter M. Anderson et al: "Oral Glutamine Reduces the Duration and severity of Stomatitis after Cytotoxic Cancer Chemotherapy", page 1433 - page 1439, abstract	13-18,20
	--	

[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
6 April 2004	27 -04- 2004
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 Terese Persson/BS Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/000098

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Lancet, Vol. 352, September 1998, Alexander P J Houdijk et al: "Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma", page 772 - page 776, abstract	13-18,20
	--	
Y	US 5462924 A (REINHOLD KIHLBERG ET AL), 31 October 1995 (31.10.1995), column 1, line 6 - line 18; column 2, line 48 - line 57	13-18,20
	--	
Y	WO 0183700 A2 (UNIVERSITY OF MARYLAND, BALTIMORE), 8 November 2001 (08.11.2001), abstract	13-18,20
	--	
Y	US 6203797 B1 (STEPHEN C. PERRY), 20 March 2001 (20.03.2001), abstract	13-18
	--	
X	US 6468525 B1 (TOMMY STANLEY WATSONET AL), 22 October 2002 (22.10.2002), abstract	19
Y		13-18
	--	
X	US 2002006432 A1 (JOHN KEVIN COLLINS ET AL), 17 January 2002 (17.01.2002), abstract; claims 15-18 and 45-54	19
	--	
X	EP 0555618 A2 (CAVALIERE VESELY, RENATA MARIA ANNA), 18 August 1993 (18.08.1993)	19
	--	
A	DATABASE WPI Week 198441 Derwent Publications Ltd., London, GB; Class B04, AN 1984-254478 & JP 59 155321 A (RYOSHOKU KENKYU-KAI), 4 September 1984 (1984-09-04)	1-20
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2004/000098

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Annales de Microbiologie, Vol. 129 B, 1978, D. Matteuzzi et al: "Amino acids produced by bifidobacteria and some clostridia", page 175 - page 181 ---	1-20
A	Agric Biol. Chem., Vol. 51, no. 1, 1987, Masayuki Hatanaka et al: "Distribution and Some Properties of Glu Synthetase and Glutamate Dehydrogenase in Bifidobacteria", page 251 - page 252 ---	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT SE 2004/000098

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7-8, 10-12 and 20 (all partly)
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.

☐ No protest accompanied the payment of additional search fees.

Box II.2

The present claims 7-8, 10-12 and 20 relate to an extremely large number of strains of Bifidobacterium due to the wordings "or a variant thereof having essentially the same REA pattern" and "or variants thereof". In fact, the claims contain so many different possible variants that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.

Consequently, the search has mainly been focused of Bifidobacterium having the ability to produce glutamine in vivo.

The present claim 20 relates to a second medical indication in which the medical conditions are vaguely defined. This lead to a lack of clarity within the meaning of Article 6 PCT to such an extent as to render a meaningful search of the whole claim impossible. The search was focused on the general definitions used in the claim as well as on the disease colitis, for which experiments has been shown in the description.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established will not be the subject of an international preliminary examination (Rule 66.1(e) PCT). This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Box III

According to PCT Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features". These special technical features are features, that define a contribution which each of the inventions make over the prior art.

The present claims 1-18 and 20 relate to a strain of Bifidobacterium having the ability to survive in the intestine tract and to produce glutamine in vivo. The present claim 19 relates to Bifidobacterium infantis for use in therapy. The common feature between these two groups of claims is the Bifidobacterium. However, these are well known, see e.g. US6468525 B1. Accordingly, there is no special technical feature apart from prior art to link the two groups of claims. The application is consequently considered to lack unity and contain the following 2 invention:

.../...

INTERNATIONAL SEARCH REPORT

International application No.
PCT SE 2004/000098

Invention 1: A strain of Bifidobacterium having the ability to survive in the intestine tract and to produce glutamine in vivo. Claims 1-18 and 20.

Invention 2: Bifidobacterium infantis for use in therapy. Claim 19.

Both of these inventions have been searched.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/02/2004

International application No.
PCT/SE 2004/000098

US	5462924	A	31/10/1995	AT	156017	T 15/08/1997
				AU	648820	B 05/05/1994
				AU	8435991	A 17/03/1992
				CA	2089257	A 25/02/1992
				DE	69127087	D,T 15/01/1998
				DK	547099	T 09/03/1998
				EP	0547099	A,B 23/06/1993
				SE	0547099	T3
				ES	2107470	T 01/12/1997
				GR	3025047	T 30/01/1998
				JP	6500109	T 06/01/1994
				SE	9002732	D 00/00/0000
				US	5646118	A 08/07/1997
				WO	9203155	A 05/03/1992
				SE	9100558	A 28/08/1992
WO	0183700	A2	08/11/2001	AU	6296601	A 12/11/2001
				CA	2407724	A 08/11/2001
				EP	1292189	A 19/03/2003
US	6203797	B1	20/03/2001	NONE		
US	6468525	B1	22/10/2002	NONE		

US	2002006432	A1	17/01/2002	AU	3071500	A	01/08/2000
				AU	3071600	A	01/08/2000
				AU	3071700	A	01/08/2000
				BR	0007481	A	09/04/2002
				BR	0007550	A	30/10/2001
				CA	2359334	A	20/07/2000
				CA	2360243	A	20/07/2000
				CN	1338940	T	06/03/2002
				CN	1342196	T	27/03/2002
				EP	1141235	A	10/10/2001
				EP	1143985	A	17/10/2001
				EP	1145001	A	17/10/2001
				ID	29150	A	00/00/0000
				ID	30449	A	00/00/0000
				IE	990033	A	18/10/2000
				IE	20000033	A	09/08/2000
				IE	20000034	A	09/08/2000
				IE	20000035	A	09/08/2000
				IL	144184	D	00/00/0000
				IL	144185	D	00/00/0000
				JP	2002534113	T	15/10/2002
				JP	2004502633	T	29/01/2004
				NO	20013429	A	27/08/2001
				NO	20013467	A	14/09/2001
				TR	200102058	T	00/00/0000
				TR	200102059	T	00/00/0000
				US	2003113809	A	19/06/2003
				US	2003166257	A	04/09/2003
				US	2003170217	A	11/09/2003
				US	2003215467	A	20/11/2003
				WO	0041707	A	20/07/2000
				WO	0042168	A	20/07/2000
				WO	0042429	A	20/07/2000
				ZA	200105616	A	25/02/2002
				ZA	200105617	A	22/10/2002
EP	0555618	A2	18/08/1993	DE	69226622	D,T	11/02/1999
				DK	555618	T	17/05/1999
				ES	2120440	T	01/11/1998
				IT	1254210	B	14/09/1995
				IT	MI920256	D	00/00/0000
				JP	6056680	A	01/03/1994
				US	5716615	A	10/02/1998