



HU000032297T2



(19) **HU**

(11) Lajstromszám: **E 032 297**

(13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 06 733290**

(51) Int. Cl.: **A61K 35/74**

(2006.01)

(22) A bejelentés napja: **2006. 04. 12.**

A61P 1/00

(2006.01)

(96) Az európai bejelentés bejelentési száma:
EP 20060733290

A61P 37/06

(2006.01)

(97) Az európai bejelentés közzétételi adatai:
EP 1868622 A1 2006. 10. 19.

(86) A nemzetközi (PCT) bejelentési szám:

PCT/SE 06/000434

(97) Az európai szabadalom megadásának meghirdetési adatai: (87) A nemzetközi közzétételi szám:
EP 1868622 B1 2016. 09. 28. **WO 06110088**

(30) Elsőbbségi adatok:
107438 **2005. 04. 15.** **US**

(73) Jogosult(ak):
BIOGAIA AB, 103 64 Stockholm (SE)

(72) Feltaláló(k):
CONNOLLY, Eamonn, S-181 30 Lidingö (SE)

(74) Képviselő:
Olle Bäcklund, Spanga

(54) **Tejsav-baktériumok emlősökben lévő gyulladások csökkentésére**

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.



(19)



(11)

EP 1 868 622 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
28.09.2016 Bulletin 2016/39

(51) Int Cl.:
A61K 35/74 (2015.01) **C12N 1/20 (2006.01)**
A61P 1/00 (2006.01) **A61P 37/06 (2006.01)**

(21) Application number: 06733290.8

(86) International application number:
PCT/SE2006/000434

(22) Date of filing: 12.04.2006

(87) International publication number:
WO 2006/110088 (19.10.2006 Gazette 2006/42)(54) **LACTIC ACID BACTERIA FOR REDUCING INFLAMMATION IN MAMMALS**

MILCHSÄURE-BAKTERIEN ZUR VERRINGERUNG VON ENTZÜNDUNGEN IN SÄUGETIEREN
BACTÉRIES D'ACIDE LACTIQUE POUR RÉDUIRE LES INFLAMMATIONS CHEZ LES
MAMMIFÈRES

(84) Designated Contracting States:
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR
HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI
SK TR**

(30) Priority: 15.04.2005 US 107438

(43) Date of publication of application:
26.12.2007 Bulletin 2007/52

(73) Proprietor: **BIOGAIA AB**
103 64 Stockholm (SE)

(72) Inventor: **CONNOLLY, Eamonn**
S-181 30 Lidingö (SE)

(74) Representative: **Owen, Deborah Jane et al**
Dehns
St Bride's House
10 Salisbury Square
London, EC4Y 8JD (GB)

(56) References cited:
WO-A1-2004/031368 US-A1- 2003 113 809

- **PENA JEREMY A ET AL: "LACTOBACILLUS REUTERI DECREASES TNF-alpha PRODUCTION IN LIPOPOLYSACCHARIDE-ACTIVATED MURINE MACROPHAGES BY A CONTACT-INDEPENDENT MECHANISM .", DIGESTIVE DISEASE WEEK ABSTRACTS AND ITINERARY PLANNER, vol. 2003, 2003, XP008143360, & DIGESTIVE DISEASE 2003; FL, ORLANDO, USA; MAY 17-22, 2003**

- **PENA J A ET AL: "Lactobacillus paracasei and Lactobacillus reuteri inhibit TNF-alpha production by Helicobacter-activated murine macrophages.", HELICOBACTER, vol. 8, no. 4, August 2003 (2003-08), page 365, XP002659907, & XVITH INTERNATIONAL WORKSHOP GASTROINTESTINAL PATHOLOGY AND HELICOBACTER; STOCKHOLM, SWEDEN; SEPTEMBER 03-06, 2003 ISSN: 1083-4389**
- **LIN YEA PING ET AL: "Probiotic Lactobacillus reuteri suppress proinflammatory cytokines via c-Jun.", INFLAMMATORY BOWEL DISEASES AUG 2008 LNKD- PUBMED:18425802, vol. 14, no. 8, August 2008 (2008-08), pages 1068-1083, XP002659908, ISSN: 1536-4844**
- **MARCEAU M. ET AL.: 'Yersinia pseudotuberculosis Anti-Inflammatory Components Reduce Trinitrobenzene Sulfonic Acid-Induced Colitis in the Mouse' INFECTATION AND IMMUNITY vol. 72, no. 4, April 2004, pages 2438 - 2441, XP003001377**
- **MENARD S. ET AL.: 'Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport' vol. 53, 2004, pages 821 - 828, XP002327491**
- **BORRUEL N. ET AL.: 'Increased mucosal tumour necrosis factor alfa production in Crohn's disease can be downregulated ex vivo by probiotic bacteria' vol. 51, 2002, pages 659 - 664, XP008023641**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

- PENA J.A. ET AL.: 'Probiotic *Lactobacillus* spp. Diminish *Helicobacter hepaticus*-Induced Inflammatory Bowel Disease in Interleukin-10-Deficient Mice' INFECTION AND IMMUNITY vol. 73, no. 2, February 2005, pages 912 - 920, XP003001378

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

Description**BACKGROUND OF INVENTION****Field of the Invention**

[0001] This description relates to use of a method for screening nonpathogenic anti-inflammatory bacterial strains, and products and methods using such strains for treatment and prophylaxis of unwanted inflammation caused by certain bacteria or other inflammation-causing agents.

Description of the Related Art

[0002] Monocytes leave the bone marrow and travel through the peripheral blood vessels until they reach the mucosa/serosa of the gastro intestinal tract. These putative macrophages are key to the interaction and propagation of the signals necessary to regulate the immune system of the GI.

[0003] In the gastrointestinal tract, there is a constant level of immune response in the macrophages of the mucosal epithelium to the bacteria in the intestinal lumen and attached to the intestinal mucosa. In the normal state, this response involves the generation of cytokine signals to restrict and contain an unnecessary inflammatory response. However, when a pathogen or toxin is presented to these cells, they form the first line of defense and react by producing an increasing amount of pro-inflammatory cytokines, which propagate the inflammatory response until the threat is removed. The generation of cytokines relevant to the interactions with commensal (non-threatening) bacteria as well as those involved in the full inflammatory response to pathogens, are subject to intervention by lactic acid bacteria themselves (including surface antigens) or by substances produced by these lactic acid bacteria and it is clear that the commensal flora has extensive interaction with the macrophages of the mucosa to maintain a balanced reaction to the gut flora and thereby maintain optimal health (Rook GA, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunopathol 2004; 25:237-255).

[0004] It is known that various pathogens can cause inflammation, for example in the gastrointestinal tract. Such inflammation, for example, in the stomach and gastrointestinal tract, is mediated by intercellular signal proteins known as cytokines which are produced by macrophages and dendritic cells in the epithelium in response to an antigenic stimulus such as that produced by a pathogen. Upon contact between the epithelium and the antigen of a pathogen or endotoxins produced by it, such as LPS, antigen presenting cells (including dendritic cells) in the epithelium propagate the signal to naive macrophages which then respond in a so-called Th-1 type response where pro-inflammatory cytokines including

TNF α , IL-1, IL-6, IL-12 are produced by the macrophages. These cytokines in turn stimulate natural killer cells, T-cells and other cells to produce interferon γ (IFN γ), which is the key mediator of inflammation. IFN γ leads to an escalation of the inflammatory response and the reactions described above that lead to cytotoxicity. Naive macrophages can also respond to antigens with a Th-2 type response. This response is suppressed by IFN γ . These Th-2 type cells produce anti-inflammatory cytokines such as IL-4, IL-5, IL-9 and IL-10.

[0005] IL-10 is known to inhibit the production of IFN γ and thus dampen the immune response. The balance between Th-1 and Th-2 type cells and their respective cytokine production defines the extent of the inflammation response to a given antigen. Th-2 type cells can also stimulate the production of immunoglobulins via the immune system. Anti-inflammatory activity in the gastrointestinal tract, where there is a reduced TNF α level, correlates with enhanced, epithelial cells (gut wall lining integrity) and thus to a reduction in the negative effects caused by gastrointestinal pathogens and toxins.

[0006] The results of a number of research studies indicate that DNA can exert an anti-inflammatory action on intestinal epithelial cells, or can stimulate the immune system (Madsen et al. and Rachmilewitz et al, respectively, presentations at Digestive Disease Week, May 19-22, 2002, The Moscone Center, San Francisco).

[0007] Inflammation is involved in several diseases in mammals both externally on skin, eyes, etc., and internally for example on various mucous membranes; in the mouth, GI tract, vagina etc. but also in muscles, bone-joints and in brain-tissue. In the GI-tract there are several diseases connected to inflammation, for example, gastritis, ulcer and inflammatory bowel disease (IBD). IBD is a chronic disorder that causes an inflamed and swollen digestive tract or intestinal wall. When the digestive tract becomes inflamed or swollen with IBD, sores (ulcers) form and bleed. This in turn can cause abdominal pain, watery diarrhea, blood in the stool, fatigue, reduced appetite, weight loss, or fever. Thus, inflammation in IBD leads to tissue damage such as ulcers and exacerbated disease in a patient.

[0008] The two most common forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Crohn's Disease is a chronic condition characterised by recurrent inflammatory lesions of the entire gut wall from mucosa to serosa and can affect many sites in the intestine. The disease has been linked to imbalances in the gut microflora and an overexpressed inflammatory reaction to components of the normal gut flora and this reaction is currently treated with poor success using a series of different drugs, one of which is based on anti-TNF α therapy designed to reduce the levels of TNF α in the gastro-intestinal mucosa. Thus, persons with such a disease form an ideal study group and indeed target group for the use of immuno-modulatory, inflammation attenuating lactobacilli.

[0009] Mice spontaneously develop chronic colitis,

which does not occur in germ-free animals. Mouse colitis is similar to human Crohn's disease, a chronic serious inflammatory disease of the gastrointestinal tract. Crohn's disease usually occurs in the intestines, but may occur anywhere in the gastrointestinal tract. These conditions require the presence of enteric bacteria and are both Th1-mediated-IL-12-dependent forms of colitis.

[0010] Because of the similarities of the causes and symptoms, mouse models of colitis and other mouse models are often used to study components of the inflammatory response directly, and, as the same mechanisms are assumed to apply in man, are often accepted to be used as models to develop treatments for human gastrointestinal disease. There are, however, some questions about the relevance of the animal derived models for humans, so there is a need to have alternative methods to study the human mechanisms and to confirm results from other models, in more human based systems. The purpose of the invention herein is to provide a method based on human cells to select lactic acid bacteria that display anti-inflammatory characteristics and then to use such selected lactic acid bacteria for prophylaxis and treatment of various inflammatory diseases.

[0011] *Lactobacillus reuteri* is one of the naturally occurring inhabitants of the gastrointestinal tract of animals and is routinely found in the intestines of healthy animals and despite the low pH, occasionally also in the human stomach. It is known to have antibacterial activity. See, for example U.S. Patent Nos. 5,439,678, 5,458,875, 5,534,253, 5,837,238, and 5,849,289. When *L. reuteri* cells are grown under anaerobic conditions in the presence of glycerol, they produce the antimicrobial substance known as reuterin (β -hydroxy-propionaldehyde). It is also known that certain strains of lactobacilli including *L.reuteri* have anti-inflammatory properties as is in U.S. patent application 20040208863 and 20040067573. Other immunomodulating activity has also been associated with various other lactobacilli.

[0012] Human clinical trials and animal studies have shown that *Lactobacillus* can prevent or improve inflammation in chronic colitis. It is hypothesised that lactobacilli are capable of down-regulating pro-inflammatory cytokine responses induced by enteric bacteria. J. Peña et al (2003) investigated whether lactobacilli diminish production of tumour necrosis factor alpha (TNF α) by a murine macrophage line. It was shown that TNF- α production by murine macrophages incubated with *Lactobacillus rhamnosus* GG and lipopolysaccharides was significantly inhibited compared to controls.

[0013] The same group of workers investigated whether *Lactobacillus* spp. might be effective in ameliorating *Helicobacter*-induced inflammation *in vivo*. For this, co-inoculation experiments with *L. reuteri* and *L. paracasei* were performed in the *H. hepaticus* mouse colitis model. A significant decrease in pathology scores was observed in female mice that have received both *H. hepaticus* and *Lactobacillus* spp. when compared to animals given *H. hepaticus* alone. The probiotic effect of *Lactobacillus* spp.

in this model was shown to be independent of the exclusion of *H. hepaticus* bacteria, but dependent on post-transcriptional regulation of TNF- α expression. The data suggests the existence of a novel mechanism by which 5 *Lactobacillus* spp. can down-regulate *Helicobacter* induced pro-inflammatory cytokine production in macrophages.

[0014] Patent WO2004/031368A1 describes *Lactobacillus* strains selected for its ability to reduce gastrointestinal inflammation associated with *H. pylori* infection in mammals using a mouse macrophage assay for TNF- α activity.

[0015] The meeting abstract by Pena Jeremy A. et al., Digestive Disease Week Abstracts and Itinerary Planner, 15 vol. 2003 & Digestive Disease 2003; Fl, Orlando, USA; May 17-22, 2003 discloses that some *Lactobacillus reuteri* strains decrease TNF-alpha production in LPS stimulated macrophages. It is suggested that enteric *Lactobacillus*-mediated inhibition of proinflammatory cytokine 20 production has an immunomodulatory effect in the gastrointestinal tract.

[0016] Patent application US20040057943A1 relates to a process for selection of new probiotic strain of *L. coryniformis*, *L. salivarius*, *L. acidophilus*, *L. gasseri* and 25 *L. fermentum* that are capable of surviving in breast milk and/or amniotic fluid.

[0017] S. Ménard et al. (2003) investigated whether lactic acid bacteria secreted metabolites that retain anti-inflammatory properties after intestinal transport. Analysis of LPS binding to THP-1 pro-monocytes in the presence of bacteria conditioned media (CM) was made to see if LPS has to bind to LPS binding protein (LBP) before LPS-LBP can recognize the CD 14 receptor. The binding was measured by flow cytometry. It was shown that *Bifidobacterium* and *Streptococcus thermophilus* CM completely inhibited LDB dependent LPS binding to THP-1 30 cells by releasing metabolites exerting an anti TNF- α effect capable of crossing the intestinal barrier.

[0018] While the differences in the ability of several 40 lactobacilli to reduce inflammation, including gastrointestinal inflammation, is known, it was not previously known that these differences are better predicted by using a model based on human cells and that such a model is preferred to select such strains for potential effect in humans.

[0019] It is therefore an object of the invention to provide a strain of lactic acid bacteria, which has been selected using human cell lines, for its capability of reducing inflammation, such as that due to IBD. It is a further object 50 of the invention to provide products containing said strain including agents for treatment or prophylaxis of inflammation, for example associated with IBD for administration to humans, including conditioned media in which said strain has grown. Thus, the invention is used to treat inflammation or ulcers caused by IBD which leads to tissue damage and exacerbated disease in a patient.

[0020] Other objects and advantages will be more fully apparent from the following disclosure and appended

claims.

SUMMARY OF THE INVENTION

[0021] The invention herein provides a strain of lactic acid bacteria selected for its capability of reducing inflammation, such as intestinal bowel disease, and products containing such a strain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022]

Figure 1 is a bar graph showing the effect of *Lactobacillus-conditioned* media on TNF- α production by LPS-activated monocytes. Three strains and controls were incubated 24 hours.

Figure 2 is a bar graph showing the effect of *Lactobacillus-conditioned* media on TNF- α production by LPS-activated monocytes. Three strains and controls were incubated 9 hours.

Figure 3 is a bar graph showing the inhibition of *Lactobacillus-conditioned* media on TNF- α production by LPS-activated primary monocytes. Primary monocytes from CD-rem (remission) and CD-act (active) patients were used. The strains and controls were incubated 24 hours.

Figure 4 is a bar graph showing the effect of *Lactobacillus-conditioned* media on TNF- α production by LPS-activated primary monocytes. Primary monocytes from CD-act patients were used. The strains and controls were incubated 24 hours.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS THEREOF

[0023] The present invention herein comprises a strain of lactic acid bacteria which has been selected for its capability of reducing inflammation, such as in IBD.

[0024] Such a strain is *Lactobacillus reuteri* MM4-1A, ATCC PTA-6475. Products such as foods, nutritional additives and formulations, pharmaceuticals or medical devices containing whole cells or components derived from this strain may be formulated as is known in the art, and generally include an ingestible support as known plus the *Lactobacillus*-strain, or its derived component. Previously known strains, now identified to have good TNF α reducing capacity, such as *L. rhamnosus* GG ATCC 53103, *L. reuteri* MM2-3, ATCC PTA-4659 and others, can also be used in above formulations.

[0025] Model systems using the appropriate cytokines are used to determine factors that reduce or increase inflammation. In the invention provided herein, an assay based on human cells is used.

[0026] THP-1 cells are a human monocytic cell line derived from leukemia patient and which are maintained at the American Type Culture Collection. The origin of these cells from a human host makes them particularly relevant

to study interactions of the human gastro-intestinal immune system with human commensal bacteria.

[0027] Data in this invention disclose an indication of a powerful inhibition of TNF α production by the specific strains *L. reuteri* ATCC PTA 4659 and *L. reuteri* ATCC PTA 6475 and that this inhibition is mediated by a substance released into the growth medium by these two specific strains during late log/stationary growth phase. On the contrary, two other strains of *L. reuteri*, were not only unable to inhibit the inflammatory response of the cells to *E. coli* toxin, but also induced an inflammatory response themselves. This surprising finding indicates a potential, anti-inflammatory properties of the strains *L. reuteri* ATCC PTA 4659 and *L. reuteri* ATCC PTA 6475 which could not be predicted.

[0028] To confirm the possible clinical relevance of these findings further studies were performed in cells derived from the blood of patients with inflammatory bowel disease, specifically Crohn's disease (CD).

[0029] To study the interactions between lactic acid bacteria and the mucosal immune cells, we considered differentiated macrophages to be a better model than undifferentiated monocytes. Such differentiated macrophages are more likely to resemble the *in vivo* macrophage cell population of the gastrointestinal tract responding to the lactic acid bacteria and eliciting the regulatory or inflammatory changes of the innate host immune system. For example, the production of pro-inflammatory interleukin-8 by THP-1 cells in response to endotoxins was markedly increased after induction of differentiation (Baqui et al., 1999) and further, TNF α production by these cells is significantly increased after differentiation (Klegeris et al., 1997).

[0030] The features of the present invention will be more clearly understood by reference to the following examples.

Example 1. Selection of anti-inflammatory strains.

[0031] THP-1 cells were incubated together with either control media or conditioned media (L-CM) from the growth of selected *L. reuteri* strains, *L. reuteri* ATCC PTA 4659, *L. reuteri* ATCC PTA 6475, *L. reuteri* ATCC 55730 and *L. reuteri* strain CF48-3A. The conditioned media (L-CM) are cell-free supernatants from 9-hour or 24-hour cultures of each of the *L. reuteri* cultures. THP-1 cells were stimulated with either control medium or *E. coli*-derived LPS (which leads to the generation of TNF α in a normal inflammatory response) during a 3.5 hour incubation after which the cells were removed and the supernatants assayed for TNF α levels using an ELISA technique.

Materials:

[0032]

THP-1 leukemic monocytic cell line (ATCC, cat

number TIB202)
 RPMI 1640 Medium (Gibco-Invitrogen)
 Fetal Bovine Serum (Gibco-Invitrogen)
 Penicillin-Streptomycin solution (Sigma)
E. coli Serotype O127:B8 Lipopolysaccharide (Sigma, cat number L3137) TNF-alpha/ TNF-SFII human DuoSet ELISA Development Kit (R&D Systems, cat number DY210)

Method:

[0033] Use THP-1 monocytic cell line. Add 5% (v/v) of MRS media and 5% (v/v) of *Lactobacillus* conditioned medium into the appropriate wells. *Lactobacillus* conditioned medium is supernatant from a 24-hour culture of *Lactobacillus* species in MRS media. The conditioned medium is then pH-adjusted by speed-vacuum drying and the pellet resuspended in equal volume of culture medium. Although the humidified chamber is designed to minimize liquid evaporation, after 48 hours of incubation, the cell suspension volume in the 24-well plates is reduced to about 475 μ l.

[0034] Add 100 ng/ml of *E. coli* serotype O127:B8 lipopolysaccharide into the appropriate wells. Incubate in a 37° C, humidified, 5% CO₂ chamber. After 3.5 hours of incubation, collect cultures into 1.5 ml centrifuge tubes. Centrifuge at 1500 RCF for 5 minutes in 4°C. Collect supernatants.

[0035] Test for cytokine expression by ELISA (Quantikine TNF-alpha/ TNF-SFII human DuoSet).

[0036] The culture medium used was 10% FBS, 2% Penicillin-Streptomycin in RPMI 1640.

Results--example 1

[0037] Incubation of THP-1 cells with 24-hour L-CM from *L. reuteri* ATCC PTA 4659, *L. reuteri* ATCC PTA 6475 strains in the absence of LPS did not lead to the generation of TNF α in the incubation medium (Fig. 1). Surprisingly, L-CM from *L. reuteri* ATCC 55730 and *L. reuteri* strain CF48-3A stimulated the production of TNF α by the THP-1 cells to levels similar to those seen with LPS alone. Thus, the *L. reuteri* strains differ in their ability to stimulate pro-inflammatory TNF α production by resting THP-1 monocytes.

[0038] Addition of LPS to the THP-1 cells in the absence of L-CM led to the generation of 138 pg/ml TNF α during the 3.5 hour incubation period. This is the expected inflammatory response of the THP-1 cells to the toxin. Addition of the growth medium (MRS), which acts as a control for the L-CM additions, led to the generation of 132 pg/ml TNF α and thus MRS did not interfere with the response to LPS. The addition of 24-hour L-CM from *L. reuteri* ATCC PTA 4659 or *L. reuteri* ATCC PTA 6475 dramatically reduced the levels of LPS stimulated TNF α to only 14 and 10 pg/ml, respectively. This represents an inhibition of LPS-stimulated TNF α production of 89 and 92%, respectively. On the contrary, in the presence of

24-hour L-CM from *L. reuteri* ATCC 55730 and *L. reuteri* strain CF48-3A, LPS was still able to induce a significant rise in TNF α compared to the levels in the absence of LPS. LPS-stimulated TNF α production increased by 50% and 38% despite the presence of L-CM from *L. reuteri* ATCC 55730 and *L. reuteri* strain CF48-3A, respectively.

[0039] Similar experiments performed with 9-hour L-CM from *L. reuteri* ATCC PTA 4659 or *L. reuteri* ATCC PTA 6475 demonstrated that the inhibitory effect on LPS-stimulated TNF α production was considerably less (52% and 16%, respectively; Fig 2) but still there. Thus, longer incubations of the *L. reuteri* strains, with harvesting of the L-CM in late log/stationary phase of growth, leads to improved efficacy in inhibiting TNF α production.

Example 2. Negative selection prep for monocyte cells

[0040] The Monocyte Isolation Kit II (Miltenyi Biotec, Inc. 12740 Earhart Avenue, Auburn, CA 95602 (800) 367-6227; <http://www.miltenyibiotec.com/index.php?>, order number 130-091-153) is an indirect magnetic labeling system for the isolation of untouched monocytes from human peripheral blood mononuclear cells (PBMC). Non-monocytic cells, i.e., T cells, B cells, NK cells, dendritic cells, and basophils are indirectly magnetically labeled using a cocktail of biotin-conjugated antibiotics against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A, and Anti-Biotin MicroBeads. In between the two labeling steps, no washing steps are required. The magnetically-labeled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled monocytes pass through the column. Isolation of relatively enriched unlabeled monocytes is achieved by depletion of the magnetically labeled cells. Level of enrichment may be assessed or confirmed by flow cytometry.

[0041] Kit Information: Monocyte Isolation Kit II (Order number 130-091-153). Kit Contents: FcR Blocking Reagent (1 ml); Biotin-Antibody Cocktail (1 ml); Anti-Biotin MicroBeads (2 ml); Materials and Equipment Not Supplied in Kit: 15 ml Corning centrifuge tubes; Refrigerated centrifuge; Pipettes and pipette tips; Serological pipetors and pipet tips; Timer; Miltenyi Biotec MACS® MS column; Miltenyi Biotec MACS® magnet; Miltenyi Biotec MACS® column stand; Hauser Scientific Bright-Line hemocytometer; Working buffer (WB): sterile 1X phosphate buffered saline (PBS, pH 7.4, Gibco catalog number 10010023) + 0.5% bovine serum albumin (BSA, Panvera catalog number P2489) + 2mM ethylenediamine-tetraacetic acid (EDTA, Gibco catalog number 15553-035)

Method (monocyte isolation kit II):

[0042] Determine the cell density by counting on a hemocytometer. Transfer cell suspension into a 15 ml Corning conical tube. Pellet cells at 300 RCF for 10 minutes in 4°C. Remove supernatant using a 10 ml serological

pipet. Resuspend cell pellet in 30 μ l of WB per 10^7 total cells. Add 10 μ l of FcR Blocking Reagent per 10^7 total cells. Add 10 μ l of Biotin-Antibody Cocktail per 10^7 total cells. Mix well and incubate for 10 minutes at 4° - 8° C. Add 30 μ l of WB per 10^7 total cells. Add 20 μ l of Anti-Biotin MicroBeads per 10^7 total cells. Mix well and incubate for an additional 15 minutes at 4° - 8° C. Wash cells by adding 10 - 20X labeling volume of WB. Centrifuge at 300 RCF for 10 minutes in 4°C. Remove supernatant using a 2 ml serological pipet. Resuspend cell pellet in 500 μ l of WB per 10^8 cells. Place MACS MS column on the magnet.

[0043] Wash column with 500 μ l of WB. Load cell suspension onto the column. Let drain through and into a collection tube (~4 min/ml). Wash column with 3 x 500 μ l of WB and let drain through into the same collection tube.

[0044] Elute captured cells into another collection tube by placing column into another tube, pipette 1 ml of WB into column and flush out cells using positive pressure with plunger supplied with the column. Push through at a rate of 2 seconds/inch.

[0045] In the above protocol it is important to work fast and use cold solutions only. Working on ice requires increased incubation times for MACS MicroBeads. Incubate in refrigerator at 4° - 8° C. Buffers or media should not contain Ca^{2+} or Mg^{2+} . BSA may be replaced by other proteins such as gelatin, human serum albumin or fetal calf serum. The type of anti-coagulant used does not affect protocol. Higher temperatures and longer incubation times may lead to non-specific cell labeling.

Example 3. Primary monocyte bioassay

[0046] Primary human monocytes were isolated from the blood of CD patients who were in a phase of active inflammation (CD-act) or were in remission (CD-rem) where the inflammation had subsided. Blood was drawn from the CD patients and peripheral blood mononuclear cells isolated. Peripheral blood monocytes were enriched using method in example 2. Suspensions of these were allowed to differentiate for 48 hours in a 37°C, humidified, 5% CO₂ chamber. After this procedure, the cells have developed into macrophages. Growth media (control) or L-CM were then added to either peripheral blood monocytes or the differentiated macrophages and the cells were stimulated with either control medium or *E. coli*-derived LPS and incubated for 3.5 hours. TNF α production in the supernatants from these incubations was analyzed.

[0047] The materials used were: Primary peripheral blood monocytes; RPMI 1640 Medium (Gibco-Invitrogen); Fetal Bovine Serum (Gibco-Invitrogen); Penicillin-Streptomycin solution (Sigma); *E. coli* Serotype O127:B8 Lipopolysaccharide (Sigma, cat number L3137); trans Retinoic Acid (CalBioChem, cat number 554720); TNF- α / TNF-SFII human DuoSet ELISA Development Kit (R&D Systems, cat number DY210).

[0048] The peripheral blood monocytes are isolated as described in the protocols: Peripheral blood mononuclear cells Isolation Protocol (described in example 2) and Monocyte Negative Selection Protocol (described in example 2). Change the cell suspension media (PBS) to cell culture media (10% FBS, 2% Penicillin-Streptomycin in RPMI 1640).

[0049] Dilute cells to 1.0×10^5 cells/ml. Plate 500 μ l cell suspension into each well of a 24-well microtiter plate. Allow cells to differentiate for 48 hours in a 37°C, humidified, 5% CO₂ chamber. Start bioassay. Add 5% (v/v) of MRS media and 5% (v/v) of *Lactobacillus* conditioned media into the appropriately wells. *Lactobacillus* conditioned media is supernatant from a 24-hour culture of *Lactobacillus* species in MRS media. The conditioned media is then pH-adjusted by speed-vacuum drying and the pellet resuspended in equal volume of culture media. Although the humidified chamber is designed to minimize liquid evaporation, after 48 hours of incubation, the cell suspension volume in the 24-well plates is reduced to about 475 - 485 μ l. Add 100 ng/ml of *E. coli* serotype O127:B8 lipopolysaccharide into the appropriate wells. Incubate in a 37°C, humidified, 5% CO₂ chamber. After 3.5 hours of incubation, collect cultures into 1.5 ml centrifuge tubes. Centrifuge at 1500 RCF for 5 minutes in 4°C. Collect supernatants. Test for cytokine expression by ELISA (Quantikine TNF- α / TNF-SFII human DuoSet).

30 Results--example 3

[0050] In primary monocytes from CD-rem patients, LPS led to the expected elevation in TNF α and this elevation could be inhibited by 43% in the presence of L-CM from *L. reuteri* ATCC PTA 6475. In primary monocytes from CD-act patients, the level of LPS-stimulated TNF α production was somewhat higher and was inhibited to the same degree by L-CM from *L. reuteri* ATCC PTA 6475 (Fig 3).

[0051] Compared to medium controls, the production of TNF α in response to LPS in differentiated macrophages derived from primary monocytes from CD-act patients, was significantly inhibited by 50% by L-CM from *L. reuteri* ATCC PTA 4659 and by 60% by L-CM from *L. reuteri* ATCC PTA 6475 (Fig. 4). Confirming the data from the THP-1 cells, L-CM derived from *L. reuteri* ATCC 55730 and *L. reuteri* strain CF48-3A were unable to inhibit the production of TNF α and on the contrary, increased the production of TNF α by 22% and 30%, respectively compared to the relevant controls (Fig. 4).

[0052] These data confirm the surprising finding that the different strains of *L. reuteri* have varying effects on TNF α production by monocytes and differentiated macrophages and that strains *L. reuteri* ATCC PTA 4659 and *L. reuteri* ATCC PTA 6475 are particularly suitable for use in the gastro-intestinal tract of humans with inflammatory bowel disease.

Example 4. Use of the Conditioned medium

[0053] Using the method in example 1, the conditioned medium from one effectively TNF α decreasing strain was selected, in this experiment the medium from *L. reuteri* ATCC PTA-4659. This medium was produced in larger scale by growing the strain in de Man, Rogosa, Sharpe (MRS) (Difco, Sparks, MD). Overnight cultures of lactobacilli were diluted to an OD₆₀₀ of 1.0 (representing approximately 10⁹ cells/ml) and further diluted 1:10 and grown for an additional 24 h. Bacterial cell-free conditioned medium was collected by centrifugation at 8500 rpm for 10 min at 4°C. Conditioned medium was separated from the cell pellet and then filtered through a 0.22 μ m pore filter unit (Millipore, Bedford, Mass.). The conditioned medium was then lyophilized and formulated, using standard methods, to make a tablet. This tablet was used as a drug by humans to effectively treat ulcer caused by IBD.

Example 5. Use of selected anti-inflammatory *Lactobacillus reuteri* strains

[0054] Using the method in example 1, the conditioned medium from one effectively TNF α decreasing strain was selected, in this experiment the medium from *L. reuteri* ATCC PTA-4659. The *L. reuteri* strain was then lyophilized and formulated, using standard methods, to make a capsule. This capsule was used as a drug by humans to effectively reduce inflammation of the mucosa in IBD patients caused by IBD.

Example 6. Characterization of protein produced by effective *Lactobacillus* strains

[0055] Different effective *Lactobacillus* conditioned media, including the *L. reuteri* strain ATCC PTA 4659, and *L. reuteri* ATCC PTA-6475 conditioned medium, were treated with various denaturing compounds to determine the nature of the putative immunomodulins derived from the bacteria. Thus, conditioned media were subjected to repetitive freeze-thawing, heat treatment, digestion with DNA digesting enzymes, proteases and inactivated proteases. The putative immunomodulin was in this way determined to be one or more proteins or peptides in nature. To determine the size of the putative protein immunomodulin, the conditioned medium was fractionated by filtration and the filtrates tested for effectiveness. In this way, the active component of the conditioned media of effective *Lactobacillus* strains was found to be approx 5 kDa in size or less.

Claims

1. A biologically pure culture of *Lactobacillus reuteri* ATCC PTA-6475.

2. A product comprising conditioned medium from *Lactobacillus reuteri* ATCC PTA-6475.
3. A biologically pure culture according to claim 1, or a product according to claim 2, for use in the treatment of ulcers caused by inflammatory bowel disease in a human patient.
4. A biologically pure culture according to claim 1, or a product according to claim 2, for use in the treatment of inflammation due to inflammatory bowel disease in a human patient.

15 Patentansprüche

1. Biologisch reine Kultur von *Lactobacillus reuteri* Stamm ATCC PTA-6475.
2. Produkt, das konditioniertes Medium von *Lactobacillus reuteri* ATCC PTA-6475 umfasst.
3. Biologisch reine Kultur nach Anspruch 1 oder Produkt nach Anspruch 2 zur Verwendung bei der Behandlung von Geschwüren, die durch eine entzündliche Darmerkrankung bei einem menschlichen Patient hervorgerufen werden.
4. Biologisch reine Kultur nach Anspruch 1 oder Produkt nach Anspruch 2 zur Verwendung bei der Behandlung einer Entzündung aufgrund einer entzündlichen Darmerkrankung bei einem menschlichen Patient.

35 Revendications

1. Culture biologiquement pure de *Lactobacillus reuteri* ATCC PTA-6475.
2. Produit comprenant un milieu conditionné à partir de *Lactobacillus reuteri* ATCC PTA-6475.
3. Culture biologiquement pure selon la revendication 1 ou produit selon la revendication 2 pour utilisation dans le traitement d'ulcères provoqués par une maladie inflammatoire de l'intestin chez un patient humain.
4. Culture biologiquement pure selon la revendication 1 ou produit selon la revendication 2 pour utilisation dans le traitement de l'inflammation due à une maladie inflammatoire de l'intestin chez un patient humain.

Fig 1

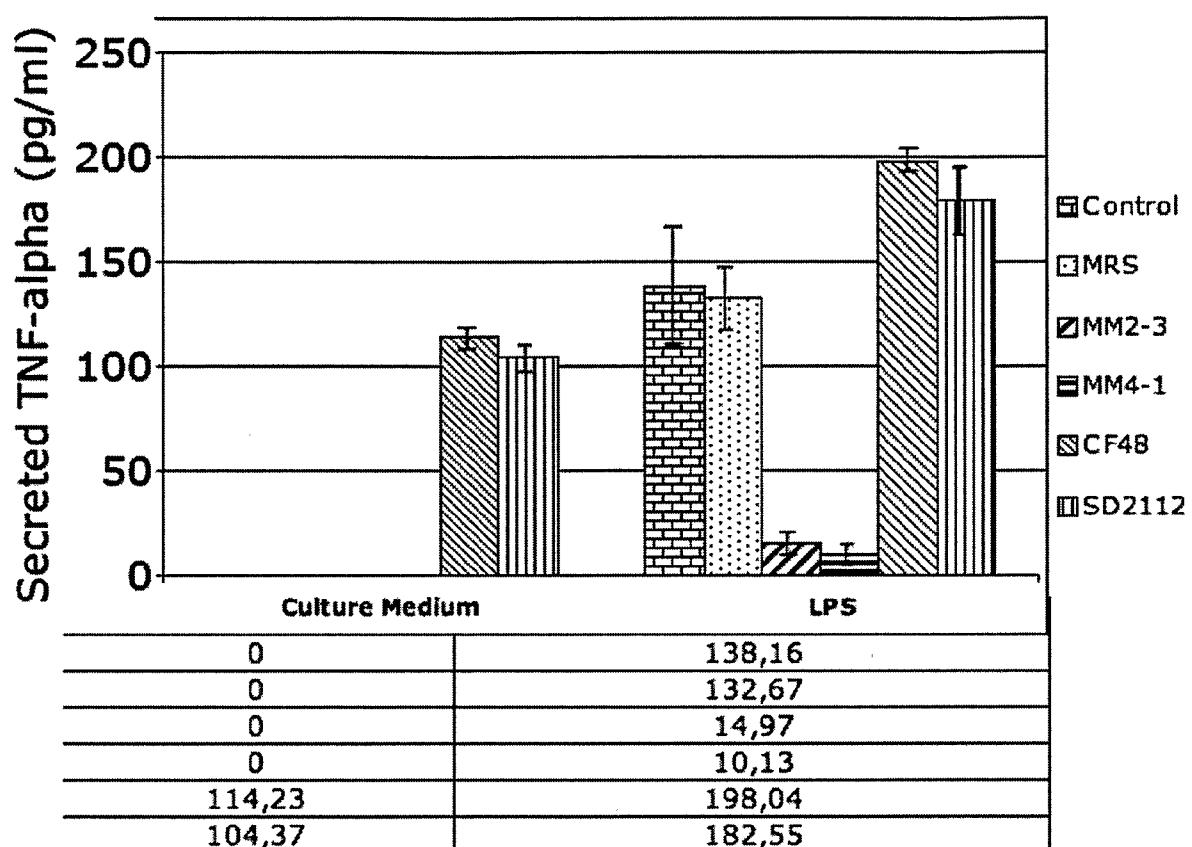


Fig 2

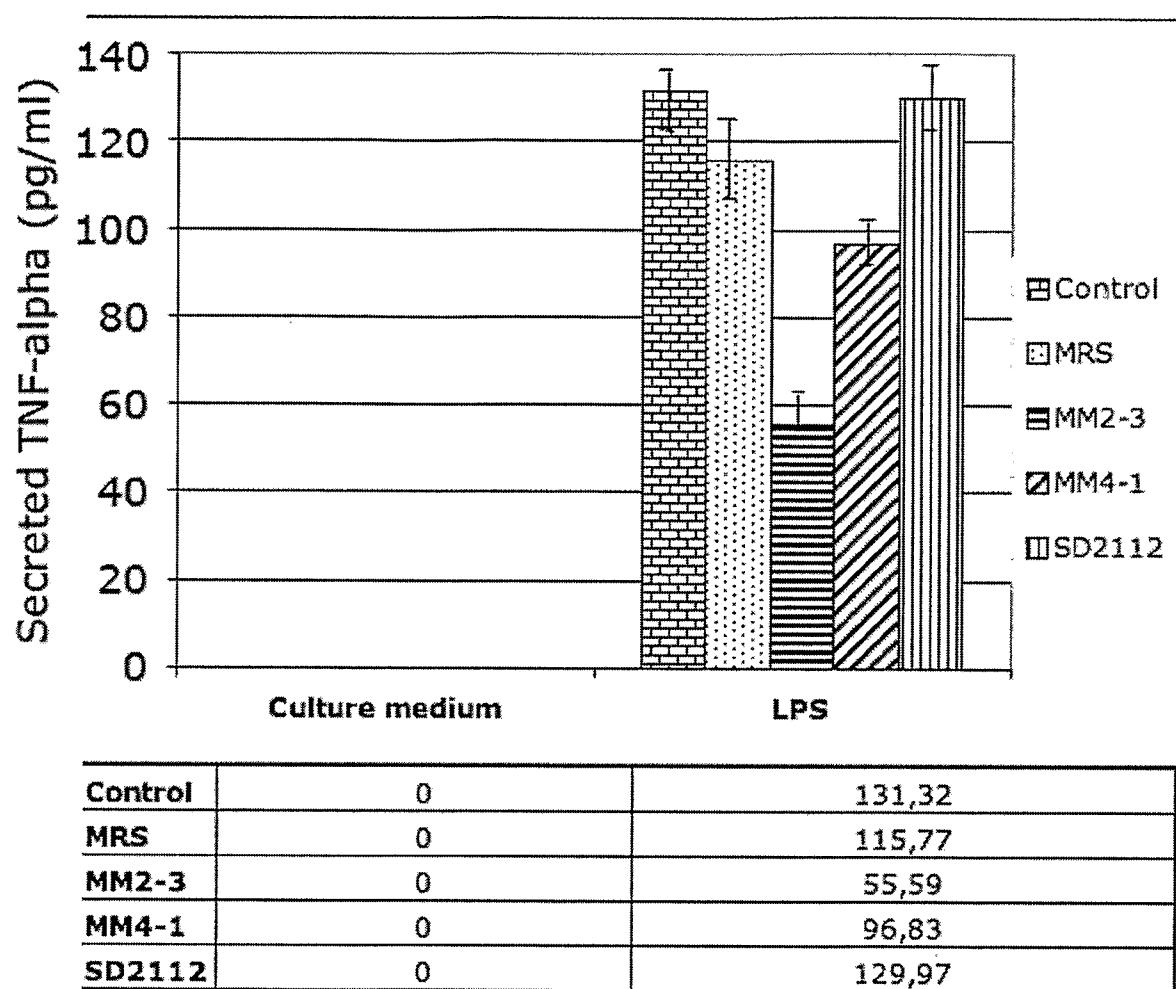
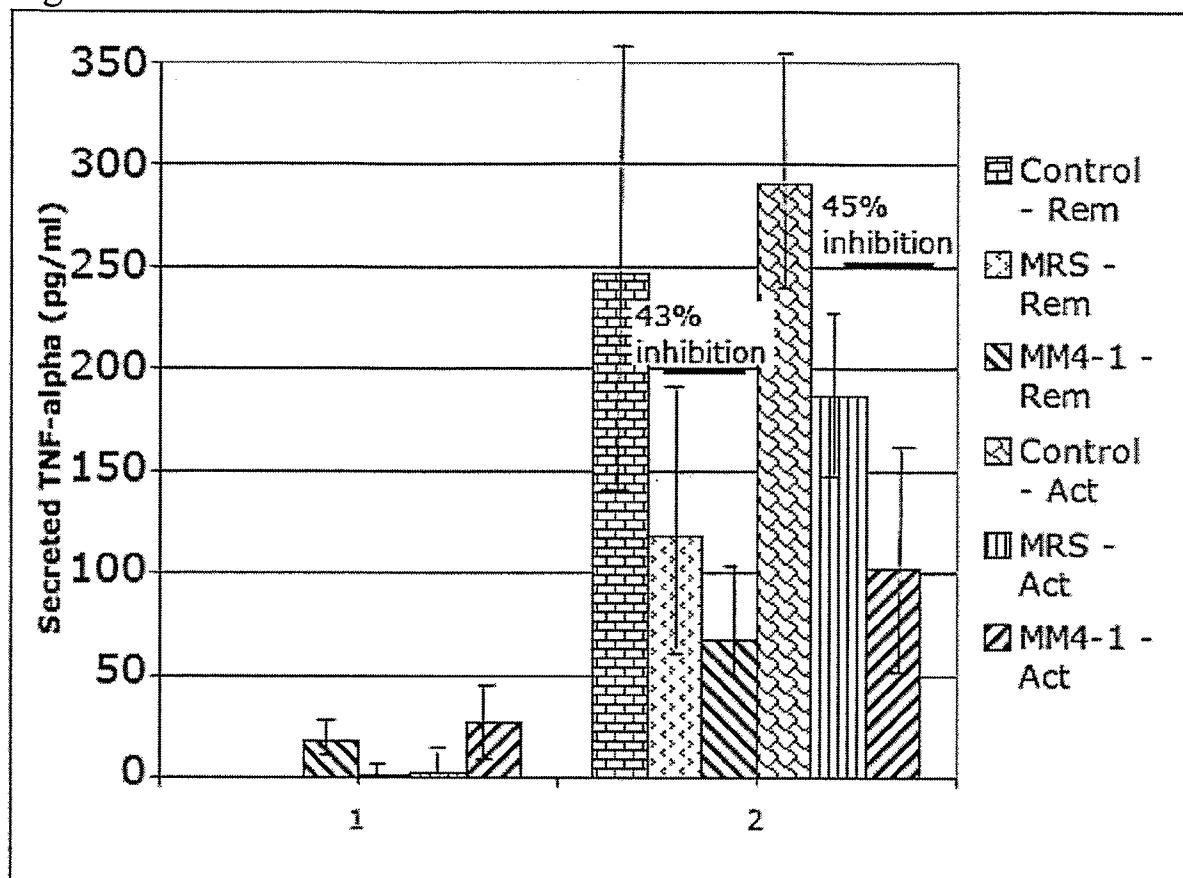


Fig.3



CD-R

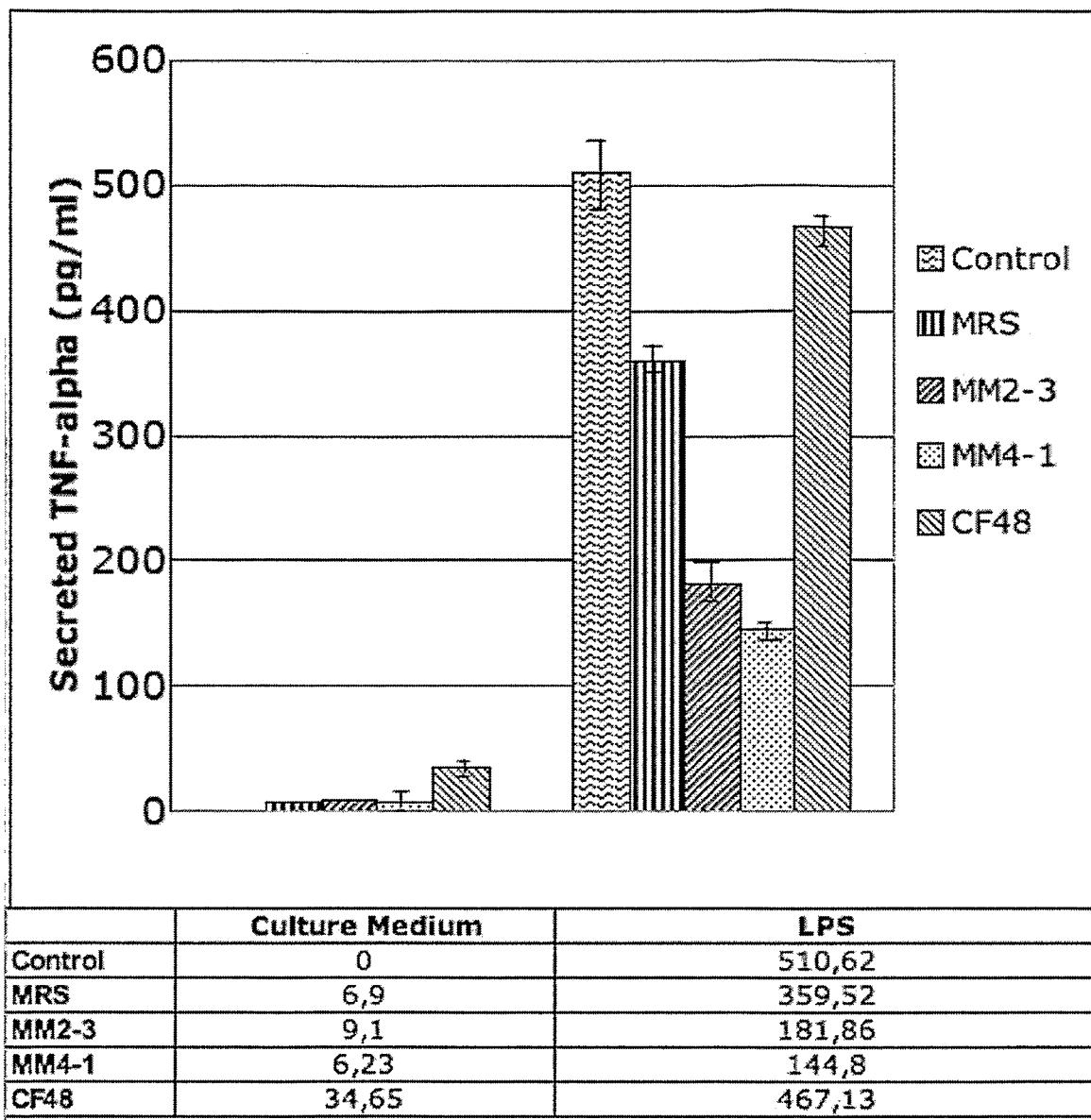
	No LPS	Control
Control-Rem	0	246,5
MRS-Rem	0	118,2
MM4-1-Rem	17,82	67,84

CD-A

	0,93	290,73
Control-Act	2,62	186,49
MRS-Act	27,3	102,04

Detection limit for TNF-a ELISA: 15 pg/ml to 1000 pg/ml

Fig.4



REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 5439678 A [0011]
- US 5458875 A [0011]
- US 5534253 A [0011]
- US 5837238 A [0011]
- US 5849289 A [0011]
- US 20040208863 A [0011]
- US 20040067573 A [0011]
- WO 2004031368 A1 [0014]
- US 20040057943 A1 [0016]

Non-patent literature cited in the description

- ROOK GA ; ADAMS V ; HUNT J ; PALMER R ; MARTINELLI R ; BRUNET LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. Springer Semin Immunopathol, 2004, vol. 25, 237-255 [0003]
- Digestive Disease Week. The Moscone Center, 19 May 2002 [0006]
- PENA JEREMY A. et al. *Digestive Disease Week Abstracts and Itinerary Planner*, vol. 2003 [0015]
- *Digestive Disease*, 2003 [0015]

SZABADALMI IGÉNYPONTOK

1. A *Lactobacillus reuteri* ATCC PTA-6475 biológiaiag tiszta tényezete.
2. Termék, amely a *Lactobacillus reuteri* ATCC PTA-6475 kondicionált médiában tartalmazza.
3. Az 1. igénypont szerinti biológiaiag tiszta kultúra vagy a 2. igénypont szerinti termék, humán páciensekben gyulladásos bélbetegség által okozott fájdalyek kezelésénél történő alkalmazásra.
4. Az 1. igénypont szerinti biológiaiag tiszta kultúra vagy a 2. igénypont szerinti termék, humán páciensekben gyulladásos bélbetegségre visszavezethető gyulladások kezelésénél történő alkalmazásra.

IS.



SZTNH-100047922