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(54) Benævnelse: **NOVEL PROBIOTIC BACTERIAL STRAIN OF LACTOBACILLUS PLANTARUM AND COMPOSITIONS AND USES THEREOF IN THE TREATMENT OF INFLAMMATION**

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EP-A1- 2 420 580
WO-A1-2010/064373
WO-A1-2014/140080
DE-U1-202009 011 379
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US-A1- 2014 023 620

DESCRIPTION

[0001] The present invention relates to a novel bacterial strain *Lactobacillus plantarum* Gos 42 (DSM 32131), and compositions thereof for use as a probiotic. The novel strain has particular use in medicine, especially in the treatment and/or prevention of inflammation. The novel strain and compositions thereof find particular utility in the treatment and/or prevention of inflammation in the oral cavity, preferably for the treatment and/or prevention of gingivitis and/or peridontitis.

[0002] In particular, the novel strain and compositions thereof can be used as anti-inflammatory agents for reducing or inhibiting the release of one or more inflammatory factors selected from the group consisting of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor (TNF), prostaglandin E2 (PGE2), isoprostanes, matrix metallopeptidase 9 (MMP9) and NF-κB.

[0003] Preferably the novel strain of the invention is a probiotic, that is, a microoganism which confers a benefit when grown in a particular microenvironment by, e.g. inhibiting or preventing the growth of other organisms within the same microenvironment. Examples of probiotic microorganisms include Lactobacilli which can colonise the gastrointestinal tract, at least temporarily, to displace or destroy pathogenic organisms, as well as providing other benefits to the host.

[0004] As utilized herein, "probiotic" refers to microorganisms that form at least a part of the transient or endogenous flora and thereby exhibit a beneficial prophylactic and/or therapeutic effect on the host organism. Probiotics are generally known to be clinically safe (i.e., non-pathogenic) by those individuals skilled in the art. By way of example, and not of limitation to any particular mechanism, the prophylactic and/or therapeutic effect of an acid-producing bacteria of the present invention results, in part, from a competitive inhibition of the growth of pathogens due to: (i) their superior colonization abilities; (ii) parasitism of undesirable microoganisms; (iii) the production of acid (e.g., lactic, acetic, and other acidic compounds) and/or other extracellular products possessing antimicrobial activity; and (iv) various combinations thereof. It should be noted that the aforementioned products and activities of the acid-producing bacteria of the present invention act synergistically to produce the beneficial probiotic effect disclosed herein.

[0005] By purified or isolated preparation of a bacterial strain is meant that the preparation does not contain another bacterial species or strain in a quantity sufficient to interfere with the replication of the preparation at a particular temperature. A purified or isolated preparation of a bacterial strain is made using standard methods, e.g. plating at limiting dilution and temperature selection.

[0006] Inflammatory conditions of the gums are primarily induced by the formation of dental plaque. Colonizing bacteria form a biofilm on the surface of the teeth aided by the presence of

food residues as well as components of saliva. If not sufficiently cleared away at an early stage, plaque films on the surface of the teeth result in deposition of dental calculus which is very hard to remove. The presence of raised numbers of bacteria at the gingival margin leads to inflammation of the gingivae, known as gingivitis. In susceptible individuals, gingivitis may progress to periodontitis, which can lead to tooth loss. In particular, lipopolysaccharides (LPS) present in Gram-negative bacteria can cause a non-specific immune response by LPS-stimulated macrophages, which release prostaglandin E2 (PEG2) and pro-inflammatory mediators such as interleukins and TNF-α in the affected tissue. The pro-inflammatory mediators induce the release of further PGE2s and matrix metalloproteinases (MMPs) from the residing fibroblasts, which destroy the extracellular matrix of the surrounding tissue. This allows bacteria to penetrate deeper into the tissue and promote the inflammatory process independent of the outer layer of the epithelium and the dental root causing the formation of a periodontal pocket. The alveolar bone supporting the tooth resorbs ahead of the advancing bacteria and, causing the tooth to become unstable and, if left untreated, lost.

[0007] In order to avoid progressive destruction of the gums, inflammatory responses in the oral cavity need to be suppressed in the early stages or ideally prevented.

[0008] Many different approaches have addressed this problem, ranging from improved methods for the mechanical removal of plaque to the use of oral care products with strong anti-bacterial properties.

[0009] However, not all the bacteria present in the oral cavity are disease-associated and many even promote oral health. Therefore, it is desirable to establish a balance towards a healthy composition of the mouth microbiota instead of non-specifically eradicating resident bacteria.

[0010] The normal oral microbiota is highly complex and includes over 700 bacterial species as well as archaea, fungi, protozoa and viruses. Lower gum commensals such as lactobacilli and bifidobacteria have been shown to have beneficial effects on gut health, including some anti-inflammatory properties, when administered as probiotics.

[0011] Probiotic action of bacteria in the oral cavity has been subject to some research but results vary strongly with the species used and suitable parameters for efficient application are hard to establish because the action may rely on largely unrelated effects.

[0012] Among the probiotic actions, general anti-bacterial effects against disease-associated species, the reduction or prevention of bacterial adhesion to the surface of the teeth as well as anti-inflammatory effects have been discussed in the literature.

[0013] EP 2420580 A1 relates to a composition comprising an effective amount of *Lactobacillus plantarum* strain CECT 7481 and *Lactobacillus brevis* strain CECT 7480, the use of said composition as a probiotic and/or a medicament for oral health applications as well as products containing said composition.

[0014] JP 2014-000039 A relates to a lactobacillus for prevention of caries, prevention/treatment of periodontal disease, improvement/prevention of foul breath, and suppression of biofilm formation in the oral cavity at ordinary times (at times other than during eating), or a culture and a culture supernatant of this lactobacillus, their neutralized products, a composition containing these, and the like. The lactobacillus is one selected from strains of *Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus sakei, Enterococcus avium,* and *Enterococcus durans.*

[0015] WO 2014/140080 A1 relates to bacterial preparations of *Lactobacillus plantarum* NCC 2936 (CNCM I-4026) and to a composition for use in improving oral health comprising a bacterial preparation of *Lactobacillus plantarum* NCC 2936.

[0016] US 2014/023620 A1 relates to probiotic compositions comprising a mixture of at least two probiotic bacteria, including *Streptococcus salivarius* K12® (BLIS K12®), such as *Streptococcus salivarius* K12® (BLIS K12®) BAA1024, and at least a *Lactobacillus* bacteria, such as one selected from those known to be suitable for oral administration, e.g. from those known to improve recipient health, particularly recipient oral health.

[0017] WO 2010/064373 A1 relates to an IL-8 inhibitor for inhibiting the production of IL-8, which comprises a culture supernatant of a plant-derived lactic acid bacterium that carries 16S rRNA gene comprising a nucleotide sequence depicted in any one of SEQ ID NO:1 to SEQ ID NO:4 or an extract from the culture supernatant.

[0018] DE 202009011379 U1 relates to probiotics-containing dentifrice and oral care preparations containing probiotic cultures from the group comprising *Bacilli, Actinobacteria, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillusjohnsonii, Lactobacillus para casej, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salvarius, Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Leuc. Mesenteroides, Ped. Acidilactici, Sporolactobacillus inulinus, Streptococcus thermophilus, Bacilus cereus, Escherichia coli, Propionibacterium freudenreichii* and *Saccharomyces cerevisiae.*

[0019] However, little is known about the influence of specific probiotic strains on inflammatory mechanisms, in particular with respect to the suppression of different inflammatory mediators. Remarkably, it has been found out in the extensive investigations leading up to the present invention, that some strains of generally acknowledged probiotic bacteria may also enhance the release of pro-inflammatory factors at certain doses. Hence, there remains an unmet need for novel strains which exhibit desirable effect on pro-inflammatory and anti-inflammatory mediators of inflammation.

[0020] An objective of the present invention is to provide a novel bacterial strain and

compositions thereof which can be used in medicine, especially in the treatment and/or prevention of inflammation, such as in the oral cavity, in particular in the treatment and/or prevention of gingivitis and/or periodontitis.

[0021] A further objective of the present invention is to provide a novel bacterial strain and compositions thereof which are capable of reducing or inhibiting the release of one or more inflammatory factors such as interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor (TNF), prostaglandin E2 (PGE2), isoprostanes, matrix metallopeptidase 9 (MMP9) and NF-κB.

[0022] The objective of the present invention is met by providing *Lactobacillus plantarum* strain GOS 42, which was deposited by Probi AB of Sölvegatan 41, 22370 Lund, Sweden at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7 B, D-38124 Braunschweig, in accordance with the requirements of the Budapest Treaty on 2 September 2015 and has received the deposit accession number DSM 32131 and compositions thereof.

[0023] The strain of the invention was isolated from the saliva of healthy human volunteers and selected from amongst over 50 candidate probiotic strains including strains of *Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifdobacterium longum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus LAFTI, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus salivarius, Streptococcus thermophilus* and *Lactococcus lactis.*

[0024] By extensive screenings, the bacterial strain according to the invention has been identified to exhibit distinct modulating activity mostly in an inhibitive manner against the release of certain pro-inflammatory factors while, at the same time, does not or only negligibly enhance the release of other pro-inflammatory factors. The present invention now allows for an optimized use of a probiotic bacterial strain for the prevention and/or treatment of inflammatory conditions, especially those occuring in the oral cavity, which has not been possible before.

[0025] As explained above, the colonization of the oral mucosa by disease-associated bacteria and the formation of plaque can tip the microbial balance in the oral cavity towards an accumulation of detrimental microorganisms, which is also referred to as dysbiosis. Therefore, the microorganism for use in the prevention and/or treatment of inflammation in the oral cavity according to the invention includes the use in the prevention and/or treatment of plaque and plaques associated diseases and advantageously aides to avoid oral dysbiosis by balancing the mouth flora towards a healthy state.

[0026] In a preferred embodiment of the present invention the microorganism described above is attenuated or dead, preferably heat-inactivated, ideally by incubation for 2 to 8 minutes at a

temperature between 70 and 100 °C.

[0027] In the studies described below, it has been demonstrated that the microorganism according to the invention can provide inhibitory effects on the release of pro-inflammatory factors even in an inactivated state. Therefore it is also possible to use the novel strain when it is dead or heat-inactivated. Remarkably, heat inactivated strains may show the same or even slightly enhanced anti-inflammatory activity towards certain factors.

[0028] In one aspect, the present invention relates to the microorganism recited above for use as anti-inflammatory agent, especially for reducing or inhibiting the release of one or more inflammatory factors selected from the group consisting of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor (TNF), prostaglandin E2 (PGE2), isoprostanes, matrix metallopeptidase 9 (MMP9) and NF-κB.

[0029] According to another aspect of the present invention, fragments of the novel strain of microorganism as defined in any of the aspects described above may be used in the treatment and/or prevention of inflammation, especially in the oral cavity, and preferably in the treatment and/or prevention of gingivitis and/or periodontitis.

[0030] It may not be necessary to use whole cells of the novel strain of the invention as mixtures comprising only fragments (e.g. debris of degraded cells) of the microorganisms are sufficient to provide the inventive effects.

[0031] In a further aspect, the present invention also relates to a pharmaceutical composition, comprising *Lactobacillus plantarum GOS 42* or fragments thereof and a pharmaceutically acceptable carrier, exipient and/or diluent. The total amount of the microorganism or fragments thereof is preferably sufficient for treating and/or preventing inflammation especially in the oral cavity, preferably for treating and/or preventing gingivitis and/or periodontitis. Preferably the total amount of the microorganism or fragments thereof is in the range from 0.01 to 100 %, more preferably in the range from 0.1 to 50 %, most preferably in the range from 1 to 10 %, in each case with respect to the total weight of the composition, and/or wherein the total amount of the microorganism(s) or the fragments thereof is in the range from 1×10^3 to 1×10^{11} colony forming units (CFU), more preferably in the range from 1×10^5 to 1×10^{10} CFU, and ideally from 1×10^8 to 1×10^9 CFU.

[0032] In a preferred embodiment, the microorganism (bacteria) of the invention is present in a composition at a concentration of approximately 1×10^3 to 1×10^{14} colony forming units (CFU) per gram, preferably approximately 1×10^5 to 1×10^{12} CFU/gram, whereas in other embodiments the concentrations are approximately 1×10^9 to 1×10^{13} CFU/gram, approximately 1×10^5 to 1×10^7 CFU/gram, or approximately 1×10^8 to 1×10^9 CFU/gram. However, it will be appreciated that is the overall CFU rather than the concentration which is most important.

[0033] In one embodiment the bacteria of the invention is in a pharmaceutically acceptable carrier or food product suitable for oral administration to mammal, preferably as a powdered food supplement, a pelletized formulation, or a liquid formulation. The mammal is preferably a human.

[0034] The skilled person is aware that the probiotic organism used in a composition or product according to the invention represent biologic material the activity of which may vary with the batch and depends also on the production or processing method. Therefore, the suitable amount can be adjusted accordingly within the given range.

[0035] Furthermore, the present invention relates to a composition or product as described above for use in medicine, preferably in the treatment and/or prevention of inflammation, especially in the oral cavity, and most preferably for use in the treatment and/or prevention of gingivitis and/or periodontitis.

[0036] A composition according to the invention may further comprise one or more components selected from the group consisting of carriers, excipients or further active ingredients such as, for example, active agents from the group of non-steroidal antiphlogistics, antibiotics, steroids, anti-TNF-alpha antibodies or other biotechnologically produced active agents and/or substances as well as analgetics, dexpanthenol, prednisolon, polyvidon iodide, chlorhexidine-bis-D-gluconate, hexetidine, benzydamine HCl, lidocaine, benzocaine, macrogol lauryl ether, benzocaine in combination with cetidyl pyridinium chloride or macrogol lauryl ether in combination with protein free hemodialysate from calf blood, as well as for example fillers (e.g. cellulose, calcium carbonate), plasticizer or flow improves (e.g. talcum, magnesium stearate), coatings (e.g. polyvinyl acetate phtalate, hydroxyl propyl methyl cellulose phtalate), disintegrants (e.g. starch, crosslinking polyvinyl pyrrolidone), softener (e.g. triethyl citrate, dibutyl phthalate) substances for granulation (lactose, gelatin), retardation (e.g. poly (meth)acrylic acid methyl/ethyl/2-trimethyl aminomethyl ester copolymerizates in dispersion, vinyl acetate/ crotonic acid copolymerizates), compaction (e.g. microcrystalline cellulose, lactose), solvents, suspending or dispersing agents (e.g. water, ethanol), emulsifiers (e.g. cetyl alcohol, lecithin), substances for modifying the rheological properties (silica, sodium alginate), substances for microbial stabilization (e.g. benzalkonium chloride, potassium sorbate), preservatives and antioxidants (e.g. DL-alpha-tocopherol, ascorbic acid) substances for modifying pH (lactic acid, citric acid), blowing agents or inert gases (e.g. fluorinated chlorinated hydrocarbons, carbon dioxide), dyes (iron oxide, titanium oxide), basic ingredients for ointment (e.g. paraffines, bees wax) and others as described in the literature (e.g. in Schmidt, Christin. Wirk- und Hilfsstoffe für Rezeptur, Defektur und Großherstellung. 1999; Wissenschaftliche Verlagsgesellschaft mbH Stuttgart oder Bauer, Frömming Führer. Lehrbuch der Pharmazeutischen Technologie. 8. Auflage, 2006. Wissenschaftliche Verlagsgesellschaft mbH Stuttgart).

[0037] A composition or product according to the present invention may also be coated or encapsulated.

[0038] Encapsulation of a composition according to the invention may have the advantage of allowing a controlled release, for example upon contact with water, or a continuous release over an extended period of time. Moreover, the composition may be protected from degradation improving the shelf life of the product. Methods for encapsulation of active ingredients are well known in the art and a number of encapsulation materials as well as methods how to apply them to a composition according to specific requirements are available.

[0039] Furthermore, a composition or product according to the invention may be in the form of a solution, suspension, emulsion, tablets, granules, powder or capsules.

[0040] The composition or product according to the invention may be selected form the group consisting of toothpaste, tooth gel, tooth powder, tooth cleaning liquid, tooth cleaning foam, mouth wash, mouth spray, dental floss, chewing gum and lozenges.

[0041] Such compositions or products may contain abrasive systems (abrasive and/or polishing components) such as silicates, calcium carbonate, calcium phosphate, aluminum oxide and/or hydroxyl apatite, surfactants such as e.g. sodium lauryl sulfate, sodium lauryl sarcosinate and/or cocamidopropyl betaine, humectants such as glycerol and/or sorbitol, thickening agents, e.g. carboxy methyl cellulose, poly ethylene glycols, carrageenans and/or Laponite®, sweeteners such as saccharine, aroma and taste correcting agents for unpleasant taste impressions, taste modifying substances (e.g. inositol phosphate, nucleotides, e.g. guanosine monophosphate, adenosine monophosphate or other substances, e.g. sodium glutamate or 2-phenoxy propionic acid), cooling agents such as menthol derivates (e.g. L-mentyl lactate, L-menthyl alkyl carbonate, menthone ketals), icilin and icilin derivates, stabilizers and active agents such as sodium fluoride, sodium monofluoro phosphate, tin difluoride, quarternary ammonium fluorides, zinc citrate, zinc sulfate, tin pyrophosphate, tin dichloride, mixtures of different pyrophosphates, triclosane, cetyl pyridinium chloride, aluminum lactate, potassium citrate, potassium nitrate, potassium chloride, strontium chloride, hydrogen peroxide, aroma substances, sodium bicarbonate and/or smell correcting agents.

[0042] Chewing gums or dental care chewing gums may comprise a chewing gum base comprising elastomers, e.g. polyvinyl acetate (PVA), polyethylene, (low or medium molecular) polyiso butane (PIB), polybutadiene, isobutene/isoprene copolymers, polyvinyl ethyl ether (PVE), polyvinyl butyl ether, copolymers of vinyl esters and vinyl ethers, styrene/butadiene copolymers (SBR) or vinyl elastomers, e.g. based on vinyl acetate/vinyl laurate, vinyl acetate/vinyl stearate or ethylene/vinyl acetate and mixtures of the mentioned elastomers as e.g. example described EP 0 242 325, US 4,518,615, US 5,093,136, US 5,266,336 US 5,601,858 or US 6,986,709. Additionally chewing gum bases may contain further ingredients, e.g. (mineral) filers, e.g. calcium carbonate, titanium dioxide, silicone dioxide, talcum, aluminum oxide, dicalcium phosphate, tricalcium phosphate, magnesium hydroxide and mixtures thereof, plasticisers (e.g. lanolin, stearic acid, sodium stearate, ethyl acetate, diacetin (glycerol diacetate), triacetin (glycerol triacetate) and trietyhl citrate), emulsifiers (e.g. phosphatides, such as lecithin and mono and diglycerides of fatty acids, e.g. glycerol monostearate), antioxidants, waxes (e.g. paraffine waxes, candelilla waxes, carnauba waxes,

microcrystalline waxes and polyethylene waxes), fats or fatty oils (e.g. hardened (hydrogenated) plant or animal fats) and mono, di or triglycerides.

Short Description of the Figures:

[0043]

Figure 1 shows the anti-Inflammatory effects of *Lactobacillus plantarum GOS* 42 (DSM 32131) in human primary monocytes on interleukin 1 beta **(A),** interleukin 6 **(B),** interleukin 8 **(C),** prostaglandin E2 **(D),** tumor necrosis factor alpha **(E)** and isoprostane (F). The left column refers to untreated cells, the right column to attenuated cells.

Figure 2 shows the anti-Inflammatory effects of *Lactobacillus plantarum GOS* 42 (DSM 32131)in attenuated form on interleukin in human gingival fibroblasts. The left column refers to interleukin 6, the right column to interleukin 8.

[0044] The following examples are added to illustrate the present invention without being intended to limit the scope.

Example 1: Establishing the cultivation and handling of probiotic strains

[0045] The strain of the invention was selected from amongst over 50 candidate probiotic strains including strains of *Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifdobacterium longum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus LAFTI, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus salivarius, Streptococcus thermophilus* and *Lactococcus lactis.*

[0046] In order to identify the optimal growth conditions and points of harvest and to determine the colony forming units (CFU) for the probiotic bacteria to be screened, first the log phase and the end of the growth phase were determined.

Bacterial growth

[0047] The frozen (-80°C) pro-biotic stocks were thawed over-night at 4°C and 6 ml of sterile 9% NaCl solution added to the 1.2 ml of bacteria at the next morning. The samples were centrifuged (5 min, 5000 rpm), the supernatant discarded, the pellet washed with 8 ml 9% NaCl and again centrifuged for 5 min at 5000 rpm. The pellet was then resuspended in 1,2 ml 9%

NaCl and 1 ml of the sample added to 50 ml 37°C warm media (MRS Bouillon, Carl Roth KG, Karlsruhe) and incubated at 37°C. The incubations were performed in a 50 ml sterile polypropylen tube (Greiner) and probes were harvested at different time points to evaluate the growth curve.

OD-determination

[0048] For determination of OD, 500 µl of the bacterial suspension were removed and diluted in 1 ml MRS Bouillon in a 1,5ml-PS-cuvette (Brand). OD-determination was performed at 600 nm (ThermoScientific, Helios Epsilon) 1.5 ml MRS Bouillon were used as blanc.

Determination of CFU

[0049] For determination of CFU, bacteria were diluted (1:10.000.000, 1:50.000.000 and 1: 100.000.000), plated on MRS-agar-plates (MRS Agar, X924, Carl Roth) and incubated for 2 days at 37°C. The grown colonies were then counted and the CFU was calculated.

[0050] The bacteria approached the log phase right from the beginning until 7 to 8 hours when they start to reach the plateau phase. The amount of bacteria to be seeded does not change the shape of the curve. 5 hours were chosen as the point at the steepest growing phase to harvest the bacteria in the log phase and 7 hours to harvest them at the end of the log phase.

Example 2: Establishing the stimulation of human monocytes with probiotics

[0051] The stimulation of the monocyte cell cultures with the novel probiotics strain was established by using the strains obtained as powder. First, several application forms such as using the grown bacteria (picked from the log-phase), the supernatant of the grown bacterial cultures, direct application of the dissolved powder and powder supernatant were tested. According to the results, the bacteria at the end of the log phase were tested this with two batches of the frozen strain. Instead of using the supernatants, heat inactivation of these two strains was established and compared the inactivated bacteria with the activated bacteria.

Measurement of cytokine; MMP-9 and PGE2 in primary human monocytes

[0052] Human primary monocytes were isolated from buffy coats of healthy human blood donors. Cells were seeded in 24-well-plates for ELISA experiments. Cells were incubated with LPS for 24 h. The probiotics (5 doses) were added 30 min before LPS treatment. After 24 h, supernatants were removed, centrifuged and investigated for IL-1beta, TNFalpha, IL-6, IL-8, MMP-9, isoprostane-8 and PGE_2 concentrations in EIAs (PGE2, from AssayDesign,

isoprostane, from Cayman) or ELISAs (all cytokines, Immunotools, MMP-9, GE Healthcare) using manufacturer's protocol. Each dose was investigated 2-3 times in two buffy coats from 2 different donors.

[0053] First, different types of probiotic lyophilized powder preparations for the stimulation of human monocytes were tested.

[0054] The probiotics were harvested and then centrifuged. The cells were dissolved in fresh media and applied to the human monocytes.

[0055] The monocytes were then incubated with the probiotics for 30 minutes, then LPS was added and after 24 hours the supernatant removed and use for the determination of the inflammatory parameters.

Example 3: Testing of heat inactivated strains

Establishment of heat inactivation

[0056] Heat inactivation was established for two batches. At the end of the bacterial growth log phase, an aliquot of the bacterial suspension was removed, added to a fresh 50 ml tube and incubated for 5 min at 80°C in a water bath. 5 minutes at 80 °C inactivated the bacteria and thus stopped their growth.

[0057] Testing the heat inactivated batches of the strain, it was found that the enhancing effects on IL-1 and TNF were not affected by heat treatment Furthermore, heat activation did not or only slightly affect PGE2 inhibition by both strains.

Example 4: Screening of the probiotics on human monocytes and effects on NF-kappaB activation

[0058] Various probiotic strains were screened in their activated and attenuated (heat-inactivated form) on LPS-induced human primary monocytes (determining IL-1beta, IL-6, IL-8, TNFalpha, PGE2, 8-isoprostane, and MMP-9.

[0059] A typical anti-inflammatory pattern of the novel strain according to Examples 1-4 is given in Figure 1.

[0060] The experiments to test effects on NF-kappaB activation induced by TNFin NIH-3T3 fibroblasts were done in a fibroblast cell line that contained stably transfected the Luciferase gene drive by a NF-kappaB dependent promoter. The cells were stimulated with TNF in the

presence or absence of the probiotics. After 6 h of stimulation the cells were lysed and the luciferase activity measured in a Luminometer.

Example 5: Screening of selected probiotics on human gingival fibroblasts

[0061] The selected strain of the invention was applied to human gingival fibroblasts. The fibroblast cultures were maintained as described in the manufacturer's protocol. Prior to stimulation, cells are seeded in 24-well plates for ELISA experiments. Cells were incubated without (unstimulated control) or with IL-1beta for 24 h. The probiotics (5 doses, depending on the outcome of screening assays) are added 30 min before IL-1 treatment. After 24 h, supernatants were removed, centrifuged and investigated for IL-6, IL-8, isoprostane, and PGE2 concentrations in EIA (PGE2, from AssayDesign, isoprostane form Cayman) or ELISA (IL-6, IL-8, Immunotools), using manufacturer's protocol. Each dose was investigated at least 2-3 times. The strains showed some IL-6 inhibiting effects.

[0062] An anti-inflammatory pattern of the strain according to Example 5 is given in Figure 2.

Example 6: Probiotic lozenge or comprimate

[0063]

No	Block	Ingredients	Isomalt Comprimates		
			Placebo	Probiotic only	+ Flavor
1	A	Magnesium Stearate	1,800%	1,800%	1,800%
2		Acesulfam	0,050%	0,050%	0,050%
3		Sucralose	0,025%	0,025%	0,025%
4		Probiotic Material		1,000%	1,000%
5		Flavor (e.g. 134229 Optamint Peppermint s/d)			0,500%
6	B	Isomalt	98,125%	97,125%	96,625%
		Sum total	**100,00%**	**100,00%**	**100,00%**

Production method:

[0064]

- components 1 and 6 are dried in a vacuum compartment drier at 50 °C and a pressure

of max. 10 mbar for 16 hours

- all components are weight out exactly
  components 1, 2, 3, 4 and 5 combined and thoroughly mixed (block A). The probiotic material is applied in lyophilized form having an activity of about 10^5 to 10^{12} colony forming units (CFU) per gram.
- block A is subsequently added to component 6 and mixed thoroughly for 5 minutes
- the powder mixture is pressed into tablets in a tablet press EK0 (Korsch AG, Berlin) at an adjusted pressure of 15 - 20 kN
  target parameters:
  - o tablet diameter: 20 mm
  - o tablet weight: 2,0 g
- storage at RT in sealed aluminum sachets. Per 5 lozenges 1 g of desiccant is used for dehumidification (activated by 3 h storage at 105°C in a vacuum compartment drier)

Example 7: Powder dentifrice

[0065]

No	Block	Ingredients	Toothpowder		
			Placebo	Probiotic only	+ Flavor
1	A	Magnesium Carbonate	3,00%	3,00%	3,00%
2		Sodium Bicarbonate	2,00%	2,00%	2,00%
3		Sodium Fluoride	0,25%	0,25%	0,25%
4		Sodium Saccharin	0,60%	0,60%	0,60%
5	B	Probiotic Material		4,00%	4,00%
6		Flavor (e.g. 134229 Optamint Peppermint s/d)			2,00%
7	c	Calcium carbonate	94,15%	90,15%	88,15%
		Sum total	**100,00%**	**100,00%**	**100,00%**

Production method:

[0066]

- component 7 is dried in a vacuum compartment drier at 50 °C and a pressure of max.

10 mbar for 16 hours

- all components are weight out exactly
- components 1, 2, 3 and 4 are combined and thoroughly mixed together (block A)
- components 5 and 6 are, if necessary, combined and thoroughly mixed (block B). The probiotic material is applied in lyophilized form having an activity of about 10^5 to 10^{12} colony forming units (CFU) per gram.
- blocks A and B are subsequently combined and thoroughly mixed together
- the mixture is added to component 7 and mixed thoroughly for 5 minutes
- the powder mixture is made up into portions of 0.5 g each storage at RT together with 1 g of desiccant per portion (activated by 3 h storage at 105°C in a vacuum compartment drier) in sealed aluminum sachets

Example 8: Powder dentifrice

[0067]

No	Block	Ingredients	**Toothpaste tablets**
1	A	Magnesium Carbonate	3,00 %
2		Sodium Bicarbonate	2,00 %
3		Sodium Fluoride	0,25 %
4		Sodium Saccharin	0,60 %
5		Sodium Laurylsulphate	0,50 %
6		Magnesium Stearate	1,00 %
7	B	Flavor (e.g. 134229 Optamint Peppermint s/d)	2,00 %
8		Probiotic Material	6,67 %
9	C	Calcium Carbonate	17,00 %
10		Microcristalline Cellulose	66,98 %
		Sum total	**100,00%**

Production method:

[0068]

- components 6, 9 and 10 are dried in a vacuum compartment drier at 50 °C and a pressure of max. 10 mbar for 16 hours.
- all components are weight out exactly

- components 1, 2, 3, 4, 5 and 6 are combined and thoroughly mixed together (block A)
- components 7and 8 are combined and thoroughly mixed together (block B). The probiotic material is applied in lyophilized form having an activity of about 10^5 to 10^{12} colony forming units (CFU) per gram.
- blocks A and B are subsequently combined and thoroughly mixed together
- components 9 and 10 are combined and thoroughly mixed together (block C)
- the two mixtures (Block A/B and Block C) are combined and mixed thoroughly for 5 minutes
- the powder mixture is pressed into tablets in a tablet press EK0 (Korsch AG, Berlin) at an adjusted pressure of 15 - 20 kN
  target parameters

  o tablet diameter: 9 mm

  o tablet weight: 0,3 g

- storage at RT in sealed aluminum sachets. Per 3 tablets 1 g of desiccant is used for dehumidification (activated by 3 h storage at 105°C in a vacuum compartment drier)

Example 9: Chewing gum

[0069]

No	Ingredients	Chewing gum with Vegetable Oil, Probiotics in Flavor		Chewing gum with Vegetable Oil, Probiotics in Oil	
1	Gum Base (e.g. Geminis T)	30,00 %	Block A	30,00 %	Block A
2	Isomalt (here: Isomalt ST-PF)	65,00 %	Block B	65,00 %	Block B
3	Sucralose coated (10% in wax)	1,00 %		1,00 %	
4	Deoiled Soy Lecithin (here: Emulpur IP)	0,30 %		0,30 %	
5	Vegetable Oil - Triglyceride	1,60 %	Block C	1,60 %	Block C
6	Probiotic Material	0,80 %	Block D	0,80%	
7	Flavor (e.g. 203191 Optamint Peppermint)	1,30 %		1,30%	Block D

Production method:

[0070]

- component 2 is dried in a vacuum compartment drier at 50 °C and a pressure of max. 10 mbar for 16 hours
- all components are weight out exactly
- component 1 is tempered to 45 - 59 °C in a chewing gum lab-kneader with the heating kneaded until a homogenous mass is obtained. The heating is on during the whole mixing process
- components 2,3 and 4 are added subsequently and kneaded until the mixture is homogenous and no powder is visible anymore
- according to the formula component 6 is either worked into component 5 (block C) or component 7 (block D). The probiotic material is applied in lyophilized form having an activity of about 10^5 to 10^{12} colony forming units (CFU) per gram. The components are mixed until an even suspension is obtained.
- First, block C is added tot he chewing gum mass and kneaded again until a homogenous mass is obtained.
- Last, block D is processed accordingly. After addition the composition has to be kneaded until an even chewing gum mass is obtained.
- the mass is taken out oft he mixer and is formed into mini-sticks by an embossing roller using the embossing set "slabs"
- storage at RT in sealed aluminum sachets . Per 7 chewing gums 1 g of desiccant is used for dehumidification (activated by 3 h storage at 105°C in a vacuum compartment drier)

Example 10: Probiotic beadlets

[0071]

components	probiotic beadlets with low load, without aroma, with dye, with gellan gum wt.%	probiotic beadlets with low load, with aroma, with dye, with gellan gum wt.%	probiotic beadlets with high load, without aroma, without dye, without gellan gum, high water content wt.%	probiotic beadlets with high load, without aroma, without dye, without gellan gum, low water content wt.%
Alginate	1,75	1,65	1,44	1,57
Gummi Arabicum	1,25	1,18	0,60	0,65
Wheat fiber	1,125	1,06	0,52	0,57

components	probiotic beadlets with low load, without aroma, with dye, with gellan gum wt.%	probiotic beadlets with low load, with aroma, with dye, with gellan gum wt.%	probiotic beadlets with high load, without aroma, without dye, without gellan gum, high water content wt.%	probiotic beadlets with high load, without aroma, without dye, without gellan gum, low water content wt.%
Dye	0,0125	0,018	-	-
Aroma	-	1,41	-	-
Glycerol	0,1875	-	-	-
probiotic	1,125	1,35	7,20	7,83
Gellan Gum	0,0625	0,059	-	-
Water	Add to 100	Add to 100	Add to 100	Add to 100
load	***approx. 20%***	***approx. 20%***	***approx. 74%***	***approx. 74%***

Production method:

[0072] Production of the calcium chloride bath for precipitation oft he alginate beadlets:

- a 2% calcium chloride solution is produces from distilled water and calcium chloride. Care has to be taken that the $CaCl_2$ is completely dissolved.

[0073] Production of the alginate solution (instead of alginate also pectin or gellan gum may be used):

- in a reaction vessel with a stirrer and which is suitable to the batch size, water is provided
- the stirrer is turned on and, while stirring at a high level, the respective amounts of alginate, gum arabicum, wheat fiber and probiotic, as well as the optionally required gellan gum are added
- the mixture is heated to 80 °C while stirring and kept at this temperature for 5 minutes - during this step the gel forming components are dissolved
- afterwards, the heating is turned off and the hot gel solution is further stirred for at least 30 minutes until it is free of lumps
- subsequently, the solution is cooled by refrigeration to 39 - 43 °C while stirring
- in a further vessel, the aroma and the dye are provided if required and thoroughly mixed In case no aroma is used, the dye is mixed with glycerol
- when the dye dispersion is mixed homogenously, it is added tot he batch vessel with the

alginate solution. The mixing vessel is washed several times with approx. 10 % of the amount of alginate solution used of water and added to the dispersion
- the alginate dispersion is stirred further for at least 5 minutes.
- Subsequently, the batch is stirred for further at least 15 minutes at a low speed to remove potentially present air.

Dripping of the alginate solution into the calcium chloride solution for precipitation of the beadlets:

[0074]

- the alginate dispersion is moved to a tightly sealable pressure stable reaction vessel having two outlets. At one outlet pressurized air is applied. The second outlet leads to the nozzles of the dripping unit via a tube.
- the reaction vessel is tempered over a heating plate so that the alginate solution reaches a temperature of approx. 45 °C. The solution is slightly stirred with a magnet stirrer.
- after application of pressure to the reaction vessel, alginate solution is pressed towards the nozzles, which are set to oscillation by an oscillator. By adaption of pressure and the frequency of the oscillator, the size oft he resulting drops at the tips of nozzles may be adjusted.
- The drops of alginate solution forming at the tips oft he nozzles fall into a collection vessel in the form of a funnel in which the calcium chloride solution prepared at the beginning circulates.
- the cured alginate beadlets pass with the calcium chloride solution through the funnel and are collected in a sieve, the collected calcium chloride solution is pumped back into the funnel below the dripping unit and thus recycled.

the beadlets are dried in an Aeromatic flowbed-drier at an supply air temperature of 80 °C until an exhaust air temperature of 45 °C is reached

REFERENCES CITED IN THE DESCRIPTION

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NY PROBIOTISK BAKTERIESTAMME AF LACTOBACILLUS PLANTARUM OG SAMMENSÆTNINGER OG ANVENDELSER DERAF TIL BEHANDLING AF BETÆNDELSE

PATENTKRAV:

1. Et isoleret præparat af en mikroorganisme, som omfatter eller består af *Lactobacillus plantarum* GOS 42 (DSM 32131).

2. En sammensætning omfattende mikroorganismen *Lactobacillus plantarum* GOS 42 (DSM 32131) sammen med bærestof, excipiens og/eller fortynder.

3. Et isoleret præparat som krævet i Krav 1 eller en sammensætning som krævet i Krav 2, hvor mikroorganismen er til stede som vegetative celler og/eller sporer.

4. Et isoleret præparat som krævet i Krav 1 eller 3 eller en sammensætning som krævet i Krav 2 eller 3, hvor mikroorganismen er svækket eller død.

5. En sammensætning som krævet i et hvilket som helst af Krav 2-4, hvor sammensætningen er en farmaceutisk sammensætning.

6. En sammensætning som krævet i et hvilket som helst af Krav 2-5, hvor den samlede mængde af mikroorganismen ligger i området fra 0,01 til 100 %, hellere i området fra 0,1 til 50 %, helst i området fra 1 til 10 %, i hvert tilfælde i forhold til den samlede vægt af sammensætningen, og/eller hvor den samlede mængde af mikroorganismen ligger i området fra 1 x 10^3 til 1 x 10^{11} kolonidannende enheder (colony forming units, CFU), hellere i området fra 1 x 10^5 til 1 x 10^{10} CFU.

7. En sammensætning som krævet i Krav 6, hvor den samlede mængde af mikroorganisme ligger i området fra 0,1 til 50% i forhold til den samlede vægt af sammensætningen; og/eller
den samlede mængde af mikroorganismen ligger i området fra 1 x 10^5 til 1 x 10^{10} CFU.

8. En sammensætning som krævet i Krav 7, hvor den samlede mængde af mikroorganismen ligger i området fra 1 til 10% i forhold til den samlede vægt af sammensætningen; og/eller
hvor den samlede mængde af mikroorganismen ligger i området fra 1 x 10^8 til 1 x 10^9 CFU.

9. En sammensætning i overensstemmelse med et hvilket som helst af Krav 2-8, hvor sammensætningen er betrukket eller indkapslet.

10. En sammensætning i overensstemmelse med et hvilket som helst af Kravene 2-9, hvor sammensætningen er i form af en opløsning, en suspension, en emulsion, en tablet, gryn, et pulver eller en kapsel.

11. En sammensætning i overensstemmelse med et hvilket som helst af Kravene 2-10, hvor sammensætningen vælges fra gruppen bestående af tandpasta, tandgel, tandpulver, tandrensevæske, tandrenseskum, mundskyl, mundspray, tandtråd, tyggegummi og pastil.

12. En sammensætning som krævet i et hvilket som helst af Kravene 2-11, hvor sammensætningen omfatter en komponent i dyrefoder og/eller -drik.

13. Et isoleret præparat eller sammensætning som krævet i et hvilket som helst foregående krav til anvendelse i medicin.

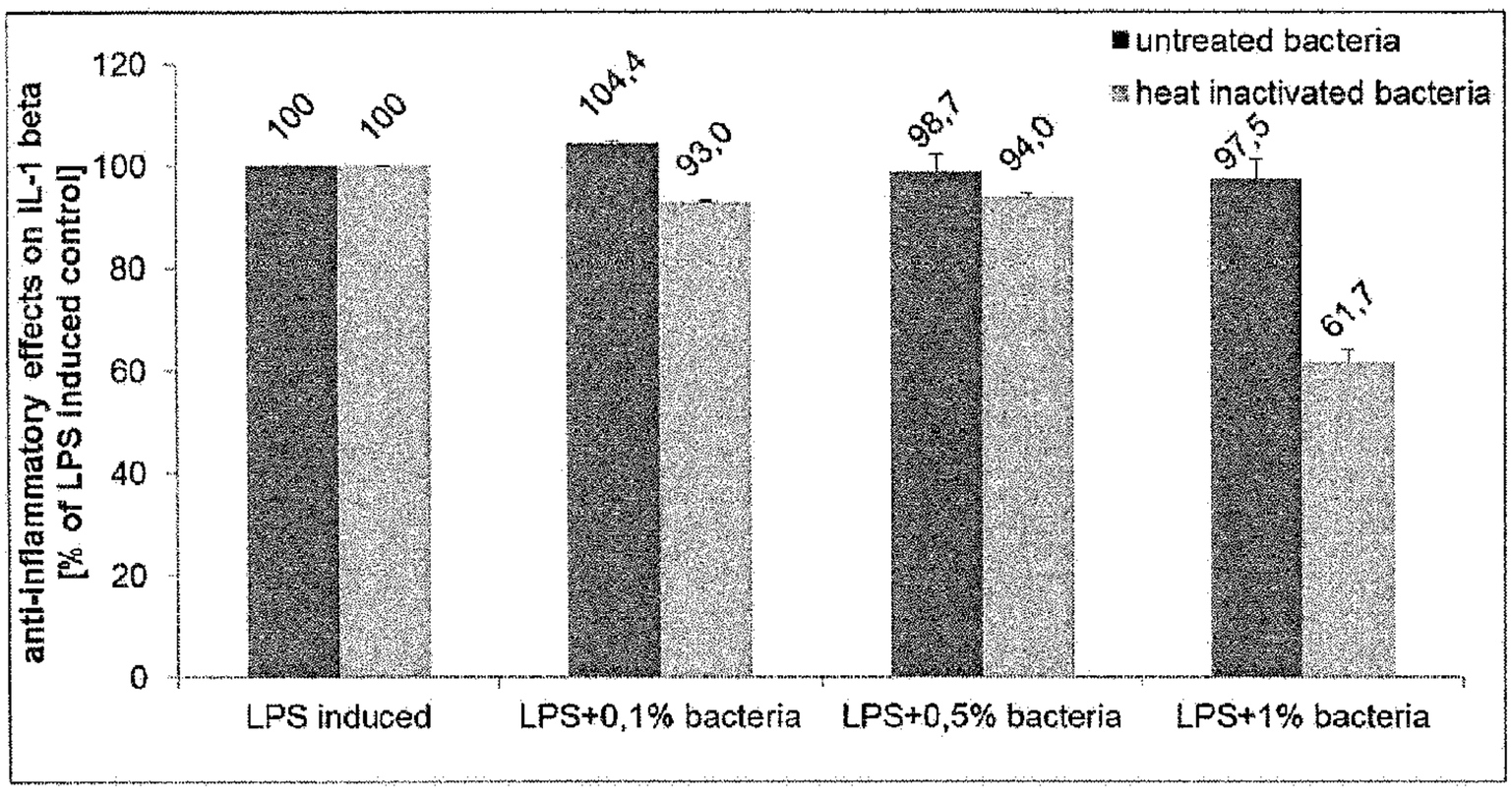
14. Et isoleret præparat eller sammensætning til anvendelse som krævet i Krav 13, hvor anvendelsen i medicin er i behandling og/eller forebyggelse af betændelse.

15. Et isoleret præparat eller sammensætning til anvendelse som krævet i Krav 13 eller 14, hvor anvendelsen i medicin er til mindskning og/eller hindring af udløsningen af én eller flere betændelsesfaktorer valgt fra gruppen bestående af interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), tumornekrosefaktor (TNF), prostaglandin E2 (PGE2), isoprostaner, matrix metallopeptidase 9 (MMP9) og NF-kB.

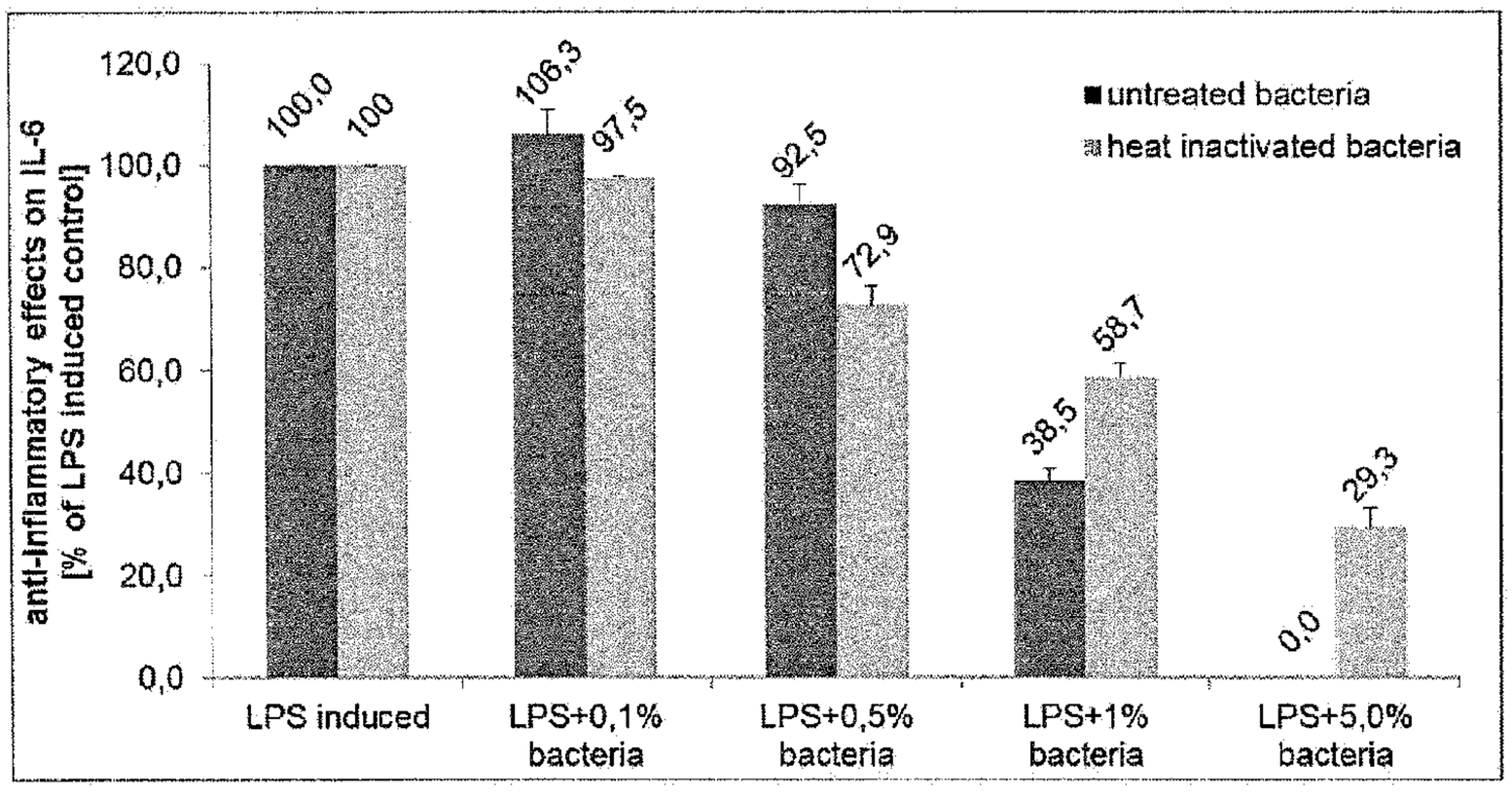
DRAWINGS

Figure 1

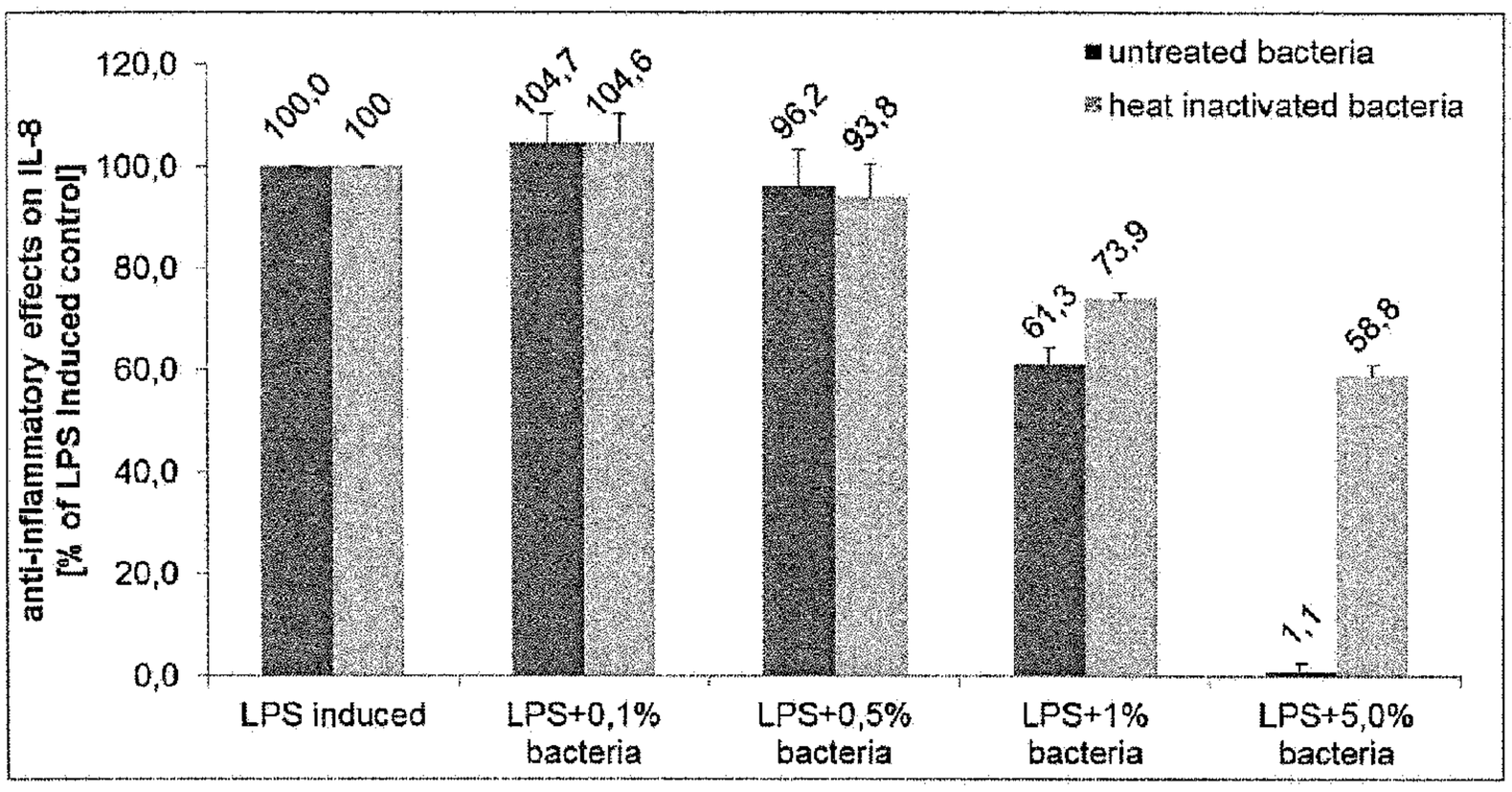
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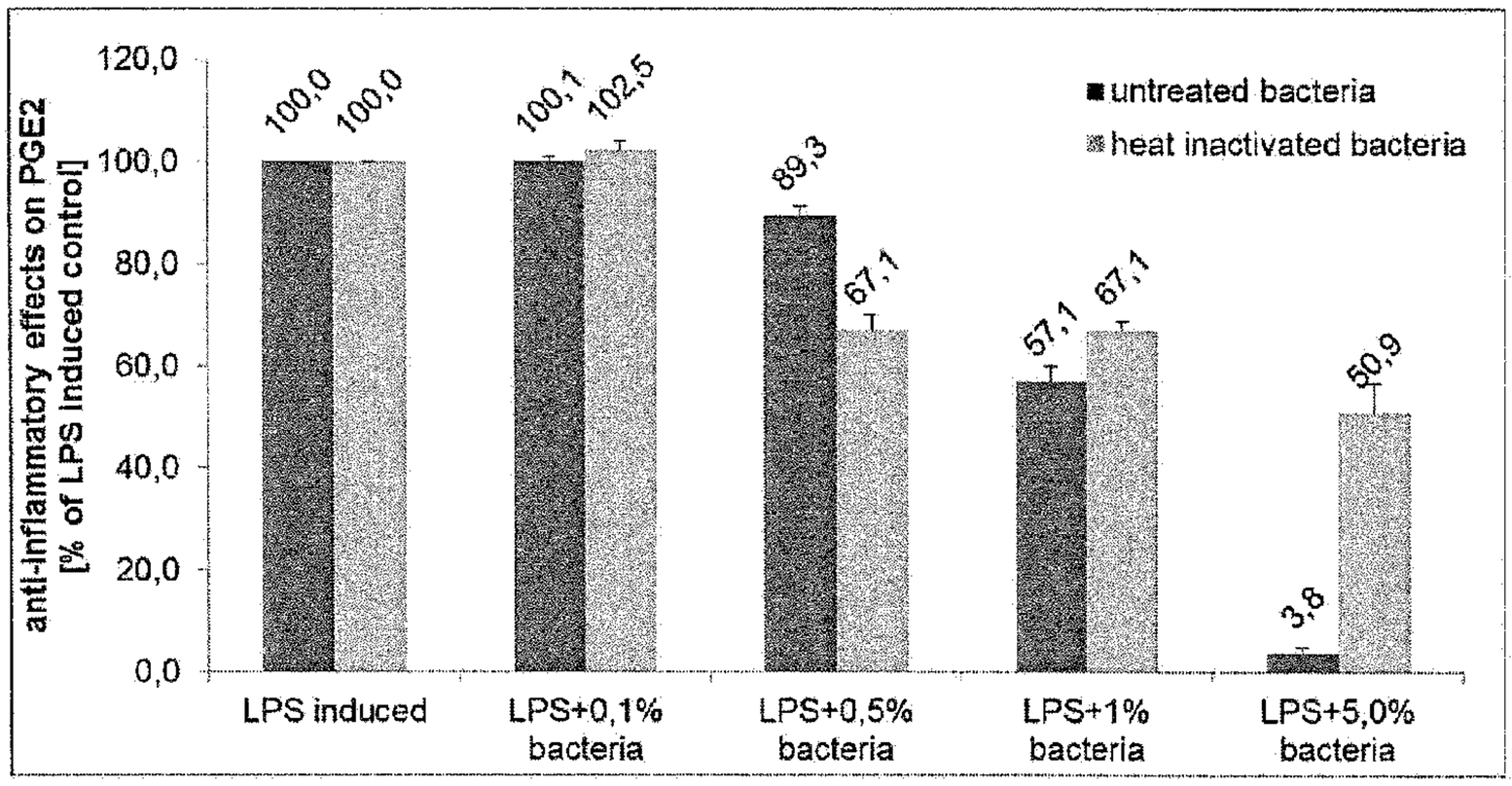
(B)



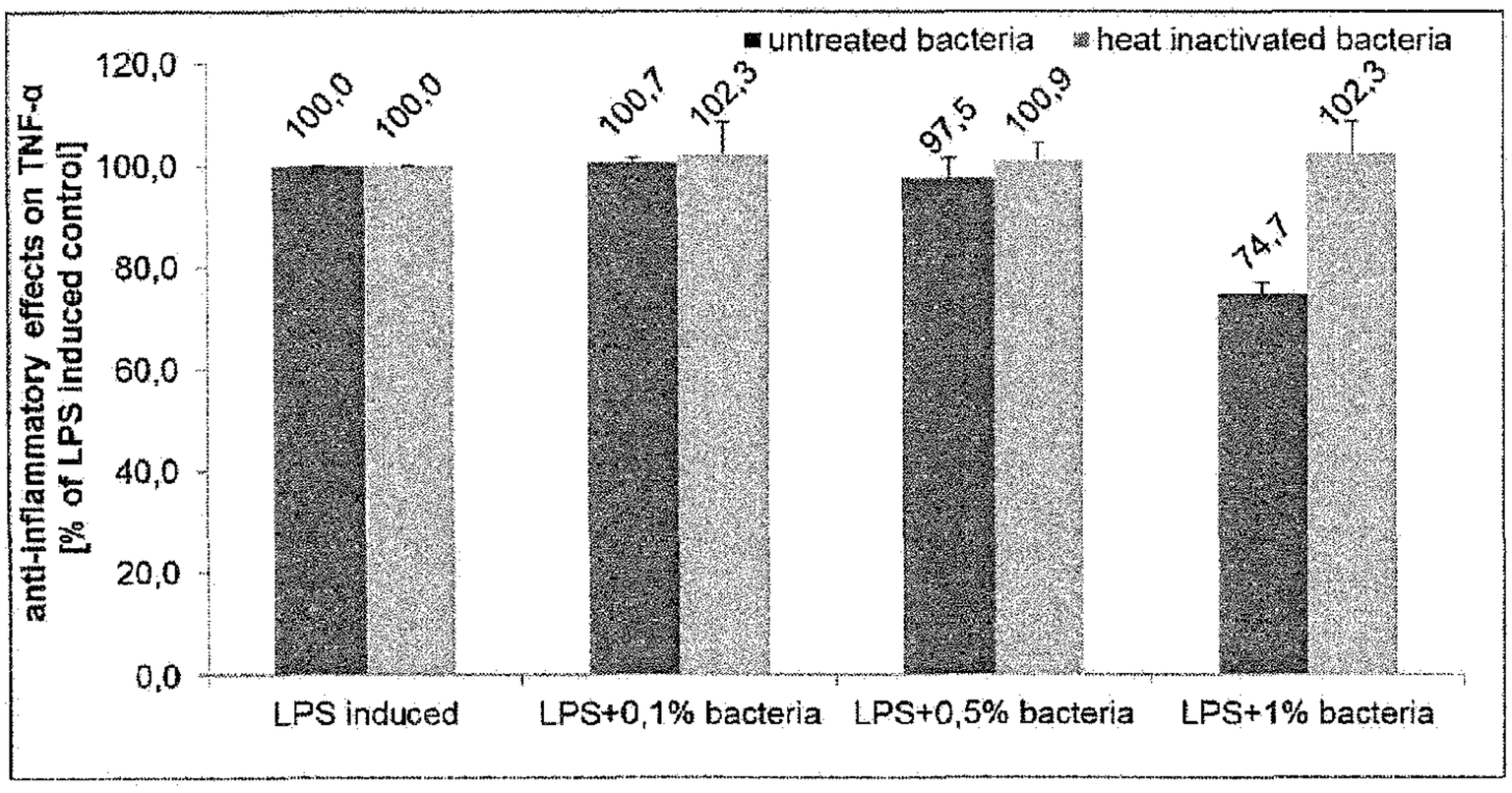
(C)


anti-inflammatory effects on IL-8
[% of LPS induced control]
120,0
100,0
80,0
60,0
40,0
20,0
0,0
100,0
100
104,7
104,6
96,2
93,8
61,3
73,9
1,1
58,8
untreated bacteria
heat inactivated bacteria
LPS induced
LPS+0,1% bacteria
LPS+0,5% bacteria
LPS+1% bacteria
LPS+5,0% bacteria

(D)


anti-inflammatory effects on PGE2
[% of LPS induced control]
120,0
100,0
80,0
60,0
40,0
20,0
0,0
100,0
100,0
100,1
102,5
89,3
67,1
57,1
67,1
3,8
50,9
untreated bacteria
heat inactivated bacteria
LPS induced
LPS+0,1% bacteria
LPS+0,5% bacteria
LPS+1% bacteria
LPS+5,0% bacteria

(E)


untreated bacteria
heat inactivated bacteria
anti-inflammatory effects on TNF-α [% of LPS induced control]
120,0
100,0
80,0
60,0
40,0
20,0
0,0
100,0
100,0
100,7
102,3
97,5
100,9
74,7
102,3
LPS induced
LPS+0,1% bacteria
LPS+0,5% bacteria
LPS+1% bacteria

(F)

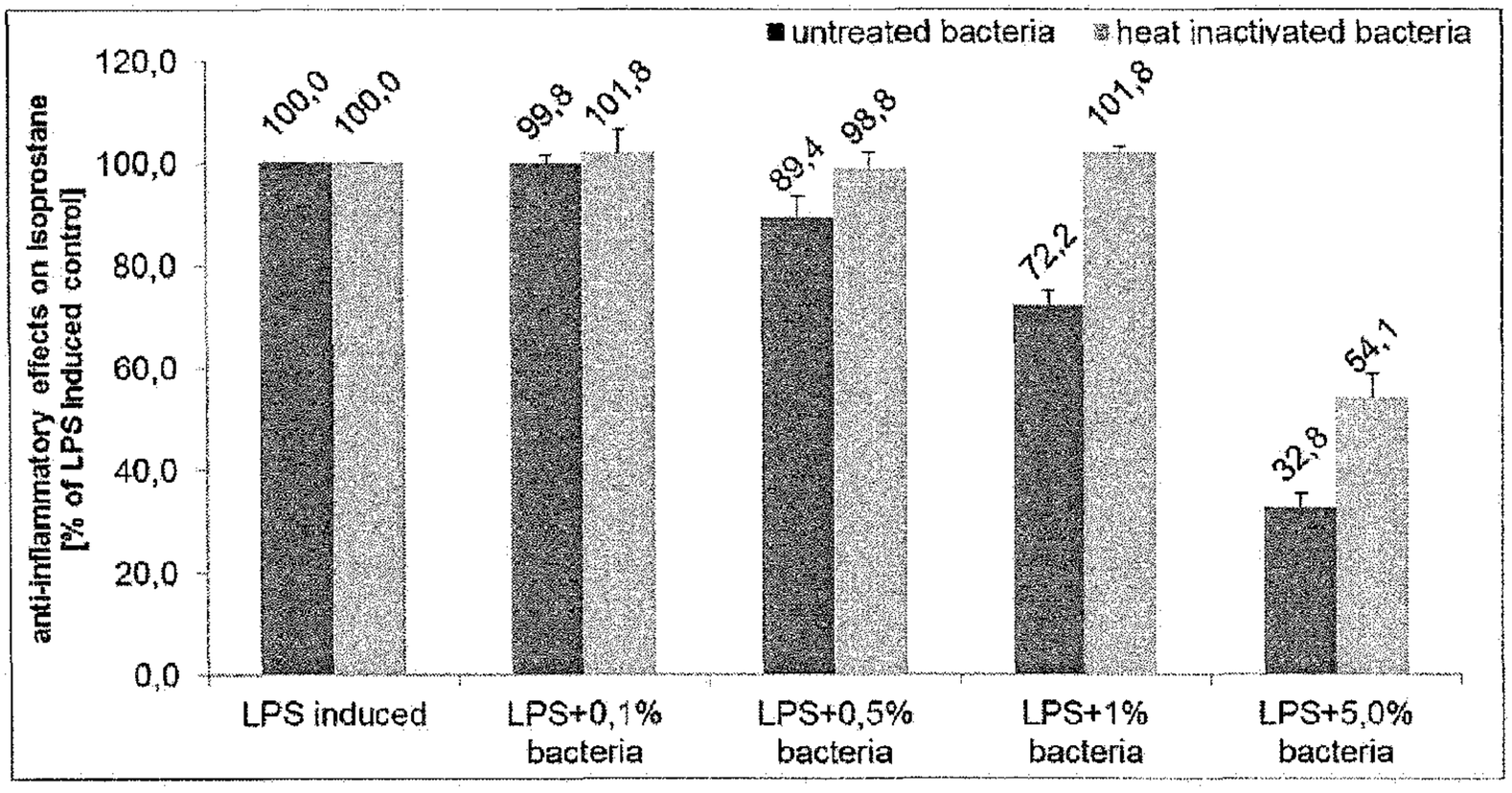
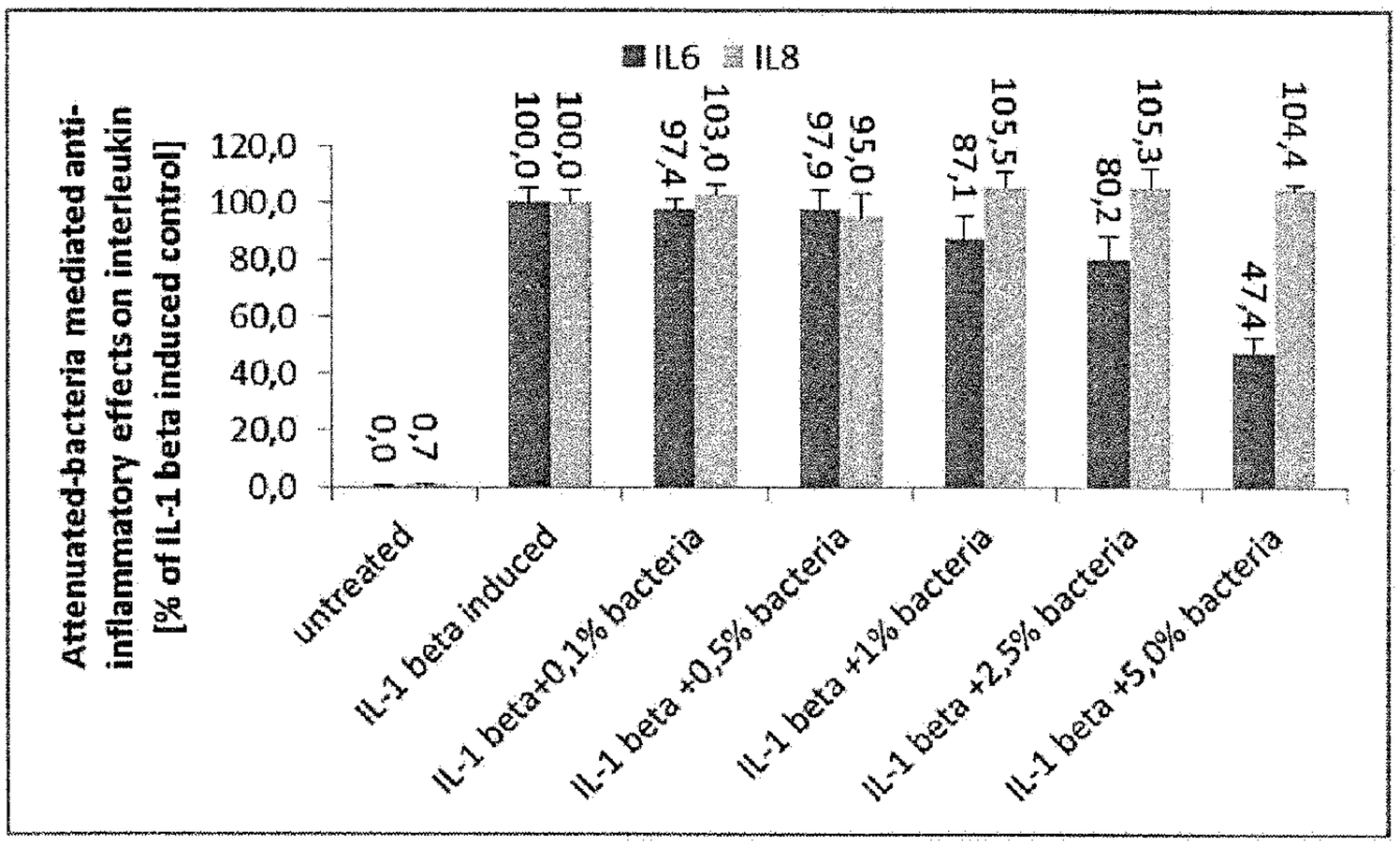

untreated bacteria
heat inactivated bacteria
anti-inflammatory effects on Isoprostane [% of LPS induced control]
120,0
100,0
80,0
60,0
40,0
20,0
0,0
100,0
100,0
99,8
101,8
89,4
98,8
72,2
101,8
32,8
54,1
LPS induced
LPS+0,1% bacteria
LPS+0,5% bacteria
LPS+1% bacteria
LPS+5,0% bacteria

Figure 2


IL6
IL8
Attenuated-bacteria mediated anti-inflammatory effects on interleukin [% of IL-1 beta induced control]
120,0
100,0
80,0
60,0
40,0
20,0
0,0
0,0
0,7
100,0
100,0
97,4
103,0
97,9
95,0
87,1
105,5
80,2
105,3
47,4
104,4
untreated
IL-1 beta induced
IL-1 beta+0,1% bacteria
IL-1 beta +0,5% bacteria
IL-1 beta +1% bacteria
IL-1 beta +2,5% bacteria
IL-1 beta +5,0% bacteria