Title: LACTIC ACID BACTERIA HAVING PRO-INFLAMMATORY CHARACTERISTICS

Abstract: The invention relates to a probiotic composition containing at least one leuconostoc strain. The invention also relates to strains, Lactococcus lactis ssp. cremoris ARH74, DSM 18891 and Leuconostoc mesenteroides ssp. cremoris PIA2, DSM 18891, which are capable of promoting Th1 type cytokines and antiviral activity. The probiotic composition of the invention is useful in dairy industry and as therapeutic substances in various health-promoting and functional food products and pharmaceuticals.
LACTIC ACID BACTERIA HAVING PRO-INFLAMMATORY CHARACTERISTICS

FIELD OF THE INVENTION

The present invention relates to lactic acid bacteria such as Lactococcus or Leuconostoc or a mixture thereof, which induce production of Th1 type cytokines from human peripheral blood mononuclear cells (PBMC) to impart an immunoregulatory function to the cells. In particular, the present invention relates to the use of Lactococcus or Leuconostoc or a mixture thereof in the preparation of a product for activating pro-inflammatory and Th1 type immune response.

The present invention relates also to a food or a nutritional product, a medical or a pharmaceutical product, and/or an animal feed, which contains said microorganism(s).

BACKGROUND OF THE INVENTION

Probiotic bacteria, such as lactobacilli, bifidobacteria and lactococci, have been shown to modulate immunological responses both in vitro and in vivo studies, with relatively little knowledge about the mechanisms that regulate the beneficial effects of probiotic bacteria at the level of host cells of the whole organism. Furthermore, there is only limited amount of comparative data between different probiotic strains.

A bacterium may be referred to as a probiotic if it essentially meets the following requirements: it remains viable in the demanding conditions prevailing in the digestive tract (low pH of the stomach, acids of the digestive system, etc.); attaches to the walls of the intestine; metabolizes in the intestine; is technologically applicable (endures processing); exhibits clinically tested and reported health effects; and is safe to consume (Lee, Y-K and Salminen, S., Trends Food Sci Technol, 6 (1995) 241-245).

The best-documented probiotics include Lactobacillus rhamnosus GG (LGG) ATCC 53103, L. johnsonii LA1, L. casei Shirotia and Bifidobacterium lactis Bb12.

The health-promoting effects of probiotics include for example balancing and maintenance of intestinal flora, stimulation of the immune system and anti-carcinogenic activity.

Immune system functions are regulated by cytokines, proteins made by cells that affect the behaviour of cells. Probiotics have been found to both
modulate the balance of pro- and anti-inflammatory cytokines and affect the generation of cell- and antibody-mediated immune response. The immunomodulatory responses are found to be strongly strain-specific. Further, no synergistic immune response effects have been generated with bacterial combinations (Fujiwara et al, Int Arch Allergy Immunol 2004, 135, 205-215).

Pro-inflammatory cytokines, such as IL-1β, IL-1α, IL-6, TNF-α, IL-12, IL-18, IFN-γ, are produced quickly and trigger the inflammation response, which is in turn followed by the production of anti-inflammatory cytokines. Anti-inflammatory cytokines, such as IL-4, IL-10, TGF-β, IL-1Ra and IL-18BP, balance the inflammation and prevent it being excessively activated.

*Lactobacillus rhamnosus* GG (LGG) has been found to increase the production of pro-inflammatory TNF-α and IL-6 as well as anti-inflammatory IL-10 in human mononuclear cells (Miettinen, M. et al., Infection and Immunity 64 (12) (1996) 5403-5). Similarly, *Lactobacillus casei* Shirotta, bifidobacteria and lactococci have been shown to be able to induce the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12.

EP 1 538 198 describes that certain microorganisms belonging to strain *Lactococcus lactis* i.e., *Lactococcus lactis* subsp. *cremoris* C60 (FERM BP 08559) and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis DRC 1 (MAFF400206), have an immunoregulatory function, which induces production of an anti-inflammatory cytokine IL-10 from mammalian dendritic or spleen cells.

The bacterial cultures most frequently used as starter cultures in the dairy industry in the manufacture of fermented milks comprise lactic acid bacteria (LAB) of the *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Streptococcus* spp. Bacteria from the genus *Leuconostoc* play an important role in the dairy industry being used in mesophilic starter cultures for the manufacture of fermented dairy products and being the dominant flavour producing strains in several cheeses. The most important function of *Leuconostoc* spp. is their ability to produce carbon dioxide (CO₂) and flavour compounds through lactose heterofermentation and citrate utilization. However, little is known about their physiological and biochemical capabilities and genetic properties. *Leuconostoc mesenteroides* ssp. *cremoris* strains are among the most useful strains for producing flavour compounds in dairy fermentations, characterized with their citrate utilization, production of diacetyl and acetoin under neutral and acidic conditions, diacetyl reductase activity, and plasmid profiles. Considerable va-
riability within strains is observed. Most strains utilized citrate under neutral conditions, at about pH 6.5, without a concomitant production of diacetyl or acetoin. Furthermore, according to the current knowledge fermented dairy products such as viscous fermented milks produced with mesophilic strains(s) cannot be considered as probiotic products.

Major problem associated with the use of the conventional mesophilic cultures, such as lactococcal species, is bacteriophages infection which is common and has a large economic impact. Some strains that are strong producers of polysaccharides, for example exopolysaccharides, are sensitive to bacteriophages as well. Consequently, dramatic adverse effect on the texture of the final fermented product can then be seen. Bacteriophages are seldom observed with *Leuconostoc* species.

Although probiotics and their effects on inflammatory function have been extensively studied, a probiotic and a composition comprising said probiotic with wide specific pro-inflammatory, anti-inflammatory, Th1 or Th2 type profiles together with balanced immune responses are still very welcome. Consequently, there is continued, evident need to offer the consumers probiotic compositions and products having clearly demonstrated balanced immunoregulatory profile which are produced in a form that allows them to be consumed as a convenient part or a supplement, for example, of the every-day diet.

Further, in the pharmaceutical field there is a continuous need for compounds and/or agents which can block, control, mitigate, or prevent the formation and/or release of cytokines from cells which produce them.

**BRIEF DESCRIPTION OF THE INVENTION**

The present invention relates to a new probiotic composition with an efficient Th1 type cytokine profile, an antiviral activity and/or a balanced immunological response, which hence is capable of enhancing natural immunity.

The present invention relates to a food or nutritional product, a medicinal or pharmaceutical product or an animal feed product comprising said probiotic composition.

The present invention further relates to strains having properties that increase Th1 type cytokine responses and antiviral activity, and hence, being well suited for use both as a starter and as a health-promoting ingredient.
In addition, the present invention relates to strains *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 and/or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 and to a bacterial starter culture and/or a probiotic composition comprising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 and/or *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891. Further, the present invention relates to a bacterial starter culture and/or a probiotic composition consisting of *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 and/or *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891, as the probiotically active strain(s).

The present invention further relates to the use of the strains *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 and/or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 in food, pharmaceutical or feed industry and to edible products, such as food and feed products and pharmaceuticals, which contain or which have been prepared by using said strain(s).

The invention further relates to the use of the probiotic composition of the invention in the preparation of a product having Th1 type immune response enhancing and antiviral characteristics.

The invention still further relates to the use of the probiotic composition of the invention, and strains *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 and/or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 in the preparation of a product for enhancing pro-inflammatory Th 1 type immune response and/or for preventing or treating allergy and/or atopic diseases and/or viral diseases and/or infectious diseases. The invention also relates to the use of the probiotic composition of the invention, and strains *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 and/or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 for enhancing pro-inflammatory Th 1 type immune response and/or for preventing or treating allergy and/or atopic diseases and/or viral diseases and/or infectious diseases.

The present invention relates also to a milk-based and/or milk-derived product as such having a probiotic effect selected from the group consisting of immunomodulation, Th1 type immune response enhancing activity, protection against allergies and/or atopic diseases and/or infectious diseases, anti-viral activity or as an ingredient for use in the preparation of functional food products.
The probiotic composition of the invention can be used as such or as a part of another product, such as a pharmaceutical or a food or a nutritional product. The composition influences the immune response by increasing the amount of Th1 type cytokines, such as IL-12 and IFN-γ. The composition of the invention is thus useful for the prevention and treatment of intestinal disorders, allergies and cancer, for fighting against viral and/or infectious diseases and for promoting general health.

Furthermore, the probiotic and/or the probiotic composition of the invention could be used in a processed milk product such as yoghurt, curdled milk, curd, sour milk, vili, buttermilk and other sour milk products. According to the invention other edible products such as milk, flavoured milk, beverages, ice-cream etc. are available. In accordance with the present invention, products are also applicable as capsules, pills or tablets that allow the use as convenient part or supplement, for example, of the every-day diet.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the production of TNF-α in *S. pyogenes* (GAS) and probiotic stimulated PBMCs with bacteria:host cell ratio of 0.2:1, 1:1 or 5:1.

Figure 2 shows the production of IL-12 in *S. pyogenes* (GAS) and probiotic stimulated PBMCs with bacteria:host cell ratio of 0.2:1, 1:1 or 5:1.

Figure 3 shows the production of IFN-γ in *S. pyogenes* (GAS) and probiotic stimulated PBMCs with bacteria:host cell ratio of 0.2:1, 1:1 or 5:1.

Figure 4 shows the production of IL-10 in *S. pyogenes* (GAS) and probiotic stimulated PBMCs with bacteria:host cell ratio of 0.2:1, 1:1 or 5:1.

**DETAILED DESCRIPTION OF THE INVENTION**

There is a continuous need for probiotics, probiotic compositions, and products having at least one probiotic effect selected from immunomodulation, Th1 type immune response enhancing activity, anti-allergic activity, anti-atopic activity, anti-infectious activity and/or anti-viral activity.

Now it has been surprisingly found that a *Leuconostoc* strain, *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892, is able to activate Th1 type immunological responses in human leukocytes.

Further, it has been surprisingly found that a *Lactococcus* strain, *Lactococcus lactis* ssp. *cremoris* ARH 74, DSM 18891, is able to activate Th1 type immunological responses in human leukocytes.
Accordingly, the present invention is directed to a probiotic composition comprising at least one *Leuconostoc* strain which is able to activate Th1 type immunological responses in human leukocytes or a composition comprising at least one *Leuconostoc* and at least one *Lactococcus* strain which are able to activate Th1 type immunological responses in human leukocytes.

The present invention is further directed to a product comprising said probiotic composition. The present invention is also directed to a product produced by using said composition. The probiotic compositions and/or products of the present invention have an effect selected from immunomodulation, Th1 type immune response enhancing activity, anti-allergic activity, anti-atopic activity, anti-infectious activity or anti-viral activity.

In one embodiment of the invention, *Leuconostoc* is *Leuconostoc mesenteroides* ssp. *cremoris*. In another embodiment of the invention, *Lactococcus* is *Lactococcus lactis* ssp. *cremoris*. In a further embodiment of the invention, *Leuconostoc mesenteroides* ssp. *cremoris* is *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, which has been deposited with the depository authority Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under accession number DSM 18892. In a still further embodiment of the invention *Lactococcus lactis* ssp. *cremoris* is *Lactococcus lactis* ssp. *cremoris*, ARH74, which has been deposited with the depository authority Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under accession number DSM 18891.

In one embodiment, the present invention is thus directed to a probiotic composition comprising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892. In another embodiment, the invention is directed to a probiotic composition comprising at least one *Leuconostoc* and *Lactococcus lactis* ssp. *cremoris* ARH 74, DSM 18891

*Lactococcus lactis* ssp. *cremoris* ARH 74 (DSM 18891) is Gram-positive, facultative anaerobe, cocci in short chains, mesophilic (with optimum temperature of about 20-25°C). The strain ferments D-galactose, D-glucose, D-fructose, D-mannose and D-lactose (bovine origin) (API 50 CHL V5).

*Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) is Gram-positive, facultative anaerobe, cocci (ovoid) in short chains, mesophilic (with optimum temperature of about 22-25°C). The strain ferments D-galactose, D-glucose and D-lactose (bovine origin) (API 50 CHL V5).
In the present invention it has surprisingly been found that said strains are able to activate Th1 type immunological responses in human leukocytes more efficiently than *Lactobacillus rhamnosus* GG (LGG), which in previous studies was found to induce strong pro-inflammatory and Th1 type responses. *Lactococcus lactis* ssp. *cremoris* ARH74 (DSM 18891) and *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) were found to be much stronger inducers of Th1 type cytokines than LGG. Furthermore, said strains were found to be much more potent inducers of pro-inflammatory responses (especially pro-inflammatory TNF-α), and Th1 type cytokines IL-12 and IFN-γ than *Streptococcus pyogenes* GAS which is a known cytokine inducer used as a positive control in the *in vitro* experiments.

The ability of the strains *Lactococcus lactis* ssp. *cremoris* ARH74 (DSM 18891) and *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) to activate pro-inflammatory and Th1 type immune response, is important and useful for example in preventing and treating allergy or an autoimmune disease atopic diseases. In allergy, the immune response is skewed towards Th2 type immune response and therefore agents probiotic bacteria and/or their specific structural components and/or secreted compounds able to balance this situation are considered to be useful in the treatment of allergy. The ability to activate pro-inflammatory and Th1 type immune response is valuable also in the treatment and prevention of mitâ?viral and/or infectious diseases.

In one embodiment of the present invention, the probiotic is and/or the probiotic composition comprises *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892). Said strain induces the production of pro-inflammatory and Th1 type cytokines TNF-α, IL-12 and IFN-γ, with the production increasing towards 24 h, indicating that it has excellent ability of inducing the production of pro-inflammatory and Th1 type cytokines. It is thus considered especially suitable for the purpose of the invention. TNF-α production was 6.1 times higher than that of LGG. Respectively, IL-12 production was 34.5 times higher and IFN-γ production 32.8 times higher than LGG’s.

In the present invention “probiotic composition” refers to orally administrable composition of metabolically active, i.e., live and/or or lyophilized, or non-viable heat-killed, irradiated or lysed probiotic bacteria. The composition may contain other ingredients.
The probiotic composition of the invention can be administered orally as such, i.e., in the form of a tablet, capsule or powder. In addition, the probiotic composition of the invention can be administered orally as a food or nutritional product, such as milk or whey based fermented dairy product, or as a pharmaceutical product.

The usual effective daily dose of a probiotic in humans is from $10^6$ to $10^{10}$ cfu.

A probiotic is optionally combined with at least one suitable prebiotic compound. A prebiotic is usually a nondigestible carbohydrate such as an oligo- or polysaccharide, or a sugar alcohol which is not degraded or absorbed in the upper digestive tract. Known prebiotics used commercial products include inulin and transgalacto-oligosaccharides.

The term "food product" is intended to cover all consumable products that can be solid, jellied or liquid, and to cover both ready-made products and products which are produced by using the probiotic composition of the invention as a starter alone or in combination with conventional starters or other probiotics. Food products can for instance be products of dairy industry or beverage industry.

In the present invention, "milk-based product" means any liquid or semi-solid milk or whey based product having a varying fat content. The milk-based product can be, e.g., cow's milk, goat's milk, sheep's milk, skimmed milk, whole milk, milk recombined from powdered milk and whey without any processing, or a processed product, such as yoghurt, curdled milk, curd, sour milk, sour whole milk, butter milk, other sour milk products, such as vill, filling of snack bars, etc. Another important group includes milk beverages, such as whey beverages, fermented milks, condensed milks, infant or baby milks; ice-cream; milk-containing food such as sweets. Another type of product of the invention is an animal feed.

In the preparation of milk-based products of the invention, conventional heat treatment methods such as pasteurization (heating for example at about 72°C for at least 15 seconds), ESL treatment (heating for example at about 130°C for 1 to 2 seconds), UHT treatment (heating for example at about 138°C for 2 to 4 seconds) or high temperature pasteurization (heating at 95°C for 5 minutes), are employed.
In one embodiment of the invention, the probiotic composition of the invention is a fermented dairy product or it is used in the preparation of a fermented dairy product.

The fermentation conditions such as, starter culture(s), temperature, pH and time for the production of fermented milk products or ingredients are selected to meet the requirements of the final product. The selection of suitable conditions belongs to knowledge of a person skilled in the art.

The fermentation is usually allowed to continue until the pH is 4.2 to 4.6. In case of fermented milk products, fermentation normally takes from 2 to 7 hours with yoghurts, up to 24 hours with sour cream or ‘villi’.

The probiotic composition of the invention and the starter, if any, are used in a balanced proportion to each other to produce the desired effect on pro-inflammatory and Th1 type cytokines. A milk-based fermented product of the invention could be produced by using the conventional fermentation procedures of the dairy industry.

Alternatively, a milk-based fermented product of the invention can be soured with a chemical acidifying agent.

The term "acidifying agent" refers to a microbiological starter or culture, a chemical acidifying agent or mixtures thereof. Acidifying may be performed by fermenting with at least one product specific culture and/or by using a chemical acidifying agent, such as organic or inorganic acid, for example.

In one embodiment of the invention, the probiotic composition is a milk-based product comprising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) and a *Lactococcus* strain. In another embodiment of the invention, the probiotic composition is a milk-based product comprising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) and *Lactococcus lactis* ssp. *cremoris* ARH 74 (DSM 18891). In a further embodiment of the invention, the probiotic composition is a milk-based product comprising a *Leuconostoc* strain and *Lactococcus lactis* ssp. *cremoris* ARH 74 (DSM 18891).

In another embodiment of the invention, the probiotic composition is a milk-based product produced by utilising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) or *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) and a *Lactococcus* strain in the preparation process. In another embodiment of the invention, the probiotic composition is a milk-based product produced by utilising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2
(DSM 18892) and Lactococcus lactis ssp. cremoris ARH 74 (DSM 18891) in the preparation process. In a further embodiment of the invention, the probiotic composition is a milk-based product produced by utilizing a Leuconostoc strain and Lactococcus lactis ssp. cremoris ARH 74 (DSM 18891).

Furthermore, the optimum conditions for an economic, inexpensive and efficient production process for producing a fermented food etc. containing an essential amount and balanced proportion of Th1 type immune response and antiviral activity characteristics is provided with said strains.

The milk-based products described above can be used as such to achieve the desired effect. Said products can also be concentrated and used as ingredients. Further, the products can also be dried and used in the form of powder or lyophilisate. The products are also applicable as capsules, pills or tablets. The products can also be used in the preparation of functional food products, health and wellness edible products, or other corresponding products. Possible forms are capsules, pills or tablets, for example, manufactured in conventional processes used in the preparation of such product for example in the pharmaceutical industry.

The cultures most frequently used in the manufacture of cultured milks are those comprising lactic acid bacteria. The industrially most useful lactic acid bacteria are found among Lactococcus species, Streptococcus species, Enterococcus species, Lactobacillus species, Leuconostoc species and Pediococcus species. Commonly used dairy starter culture strains of lactic acid bacteria are generally divided into mesophilic organisms having optimum growth temperatures at about 30°C and thermophilic organisms having optimum growth temperatures in the range of about 40 to about 45°C. Typical organisms belonging to the mesophilic group include Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Leuconostoc mesenteroides subsp. cremoris, Pediococcus pentosaceus, Lactococcus lactis subsp. lactis biovar. diacetylactis and Lactobacillus casei subsp. casei. Thermophilic lactic acid bacterial species include as examples Streptococcus thermophilus, Enterococcus faecium, Lactobacillus lactis, Lactobacillus helveticus, Lactobacillus delbrueckii subsp. bulgaricus and Lactobacillus acidophilus.

However, even today the exact composition or the ratio of the starter cultures based on the strain(s) level is not always known. Furthermore, the ratio of different strains is significant for the characteristics of the final fermented product. For example, in case of vili, typically 3 to 4 acid forming lactic
acid bacteria strains together with one flavour forming lactic acid bacteria strain are used in the dairy industry.

The probiotic composition of the invention having pro-inflammatory and Th1 type characteristics can be added to a food product during its preparation or to a finished food product. The food products in question thus contain the desired cytokines.

The form of each of the food product, food material, and/or the pharmaceutical products, and the animal feed is not particularly limited. Examples of suitable food and/or nutritional products are dairy products, drinks, juices, soups or children's foods.

In one embodiment of the invention the probiotic composition of the invention is used to ferment milk or milk-based liquid or milk-derived liquid such as, whey, cheese whey, acid whey.

The probiotic composition and the products of the invention are primarily suitable for use for human adults and infants. The positive effects of the products are also beneficial to animals, especially pets and production animals. Examples of these include dogs, cats, rabbits, horses, cows, pigs, goats, sheep and poultry.

The following examples illustrate the present invention. The examples are not to be construed to limit the claims in any manner whatsoever.

**Example 1. In vitro immunomodulatory potential of strains**

Cytokine gene expression patterns in human peripheral blood mononuclear cells (PBMC) in response to stimulation with different probiotic bacterial strains were analyzed.

**Bacterial strains**

*Lactococcus lactis* ssp. cremoris ARH74 (DSM 18891) and *Leuconostoc mesenteroides* ssp. cremoris PIA2 (DSM 18892) were stored in skimmed milk at -70°C and passaged three times before used in stimulation experiments. ARH74 was grown in calciumcitrate-mediumagar and M17-medium. PIA2 was grown in MRS-medium. *Lactobacillus rhamnosus* GG (LGG) (ATCC 53103) was grown in MRS-medium. For stimulation experiments bacteria were grown to logarithmic growth phase, and the number of bacterial cells was determined by counting in a Petroff-Hauser counting chamber.

*Streptococcus pyogenes* (GAS) serotype T1M1 (IH32030) (National Public Health Institute, Helsinki, Finland) was used as a positive control. It was grown in TY-medium supplemented with 0.2% of glucose.
Cell culture
Human peripheral blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation over Ficoll-paque gradient (Amersham-Pharmacia Biotech, Uppsala, Sweden) as described by Pirhonen et al. (J. Immunol. 162 (1999) 7322-7329).

Stimulation experiments
Stimulations were carried out in RPMI-1640 medium containing 10% FCS, with optimal bacteria:host cell ratio of 1:1. To characterize cytokine production pattern induced by probiotic bacteria, PBMC were stimulated with probiotics and cell culture supernatants were collected at different time points after stimulation. Supernatants were stored at -20°C and used for cytokine quantification by enzyme-linked immunoabsorbent (ELISA) as described by Miettinen et al., Infect Immunol 66 (1998) 6058-6062.

Cytokine specific ELISAs
TNF-α and IL-10 were determined with antibody pairs and standards obtained from BD Pharmingen (San Diego, CA, USA) with the procedures and the conditions followed the protocol attached to the kit. IFN-γ and IL-12p70 were determined with Eli-pair kits (BioSite, Täby, Sweden) in accordance with their protocol.

Results
Results are expressed as means ± SD. Cell culture supernatants from bacteria stimulated supernatants were collected and cytokine levels measured with ELISA.

The ability of strains to induce cytokine responses was strongly strain dependent. Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892) was the best inducer of pro-inflammatory and Th1 type cytokines TNF-α, IL-12 and IFN-γ, with the production increasing towards 24 h, indicating being extremely excellent in the ability of the inducing the production of pro-inflammatory and Th1 type cytokines.

Lactococcus lactis ssp. cremoris ARH74 (DSM 18891) induced 1.3 times higher TNF-α production than the positive control Streptococcus pyogenes (GAS) (3346 vs. 2520 pg/ml) and 3.3 times higher than the reference bacterium Lactobacillus rhamnosus GG (LGG) (3346 vs. 1009 pg/ml). Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892) induced 2.5 times higher TNF-α production than the positive control GAS (6176 vs. 2520 pg/ml)
and 6.1 times higher than the reference bacterium LGG (6176 vs. 1009 pg/ml). The results are shown in Table 1 and Figure 1.

**Table 1.** Dose-dependent TNF-α production (pg/ml). Bacteria:host cell ratio of 0.2:1, 1:1 or 5:1 was used. Cell culture supernatants from bacteria stimulated supernatants were collected and cytokine levels measured with ELISA. Results are mean ± SD from four different blood donors.

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<th>5:1</th>
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<tr>
<td>GAS</td>
<td>2683 ± 1101</td>
<td>2520 ± 825</td>
<td>1211 ± 659</td>
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<td>LGG</td>
<td>814 ± 576</td>
<td>1009 ± 413</td>
<td>471 ± 191</td>
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<td>ARH74</td>
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<td>PIA2</td>
<td>5496 ± 2618</td>
<td>6176 ± 2877</td>
<td>3989 ± 1215</td>
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*Lactococcus lactis* ssp. *cremoris* ARH74 (DSM 18891) induced 7.5 times higher IL-12 production than the reference bacterium LGG (15 vs. 2 pg/ml). *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) induced 34.5 times higher IL-12 production than the reference bacterium LGG (69 vs. 2 pg/ml). The results are shown in Table 2 and Figure 2.

**Table 2.** Dose-dependent IL-12 production (pg/ml). Bacteria:host cell ratio of 0.2:1, 1:1 or 5:1 was used. Cell culture supernatants from bacteria stimulated supernatants were collected and cytokine levels measured with ELISA. Results are mean ± SD from four different blood donors.

<table>
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<tr>
<th></th>
<th>0.2:1</th>
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</table>
Lactococcus lactis ssp. cremoris ARH74 (DSM 18891) induced 17.3 times higher IFN-γ production than the reference bacterium LGG (1384 vs. 80 pg/ml). Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892) induced 32.8 times higher IFN-γ production than the reference bacterium LGG (2624 vs. 80 pg/ml). The results are shown in Table 3 and Figure 3.

Table 3. Dose-dependent IFN-γ production (pg/ml). Bacteria:host cell ratio of 0.2:1, 1:1 or 5:1 was used. Cell culture supernatants from bacteria stimulated supernatants were collected and cytokine levels measured with ELISA. Results are mean ± SD from four different blood donors.

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<td>LGG</td>
<td>526 ± 554</td>
<td>80 ± 93</td>
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<td>ARH74</td>
<td>1229 ± 1035</td>
<td>1384 ± 1523</td>
<td>61 ± 44</td>
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<tr>
<td>PIA2</td>
<td>4090 ± 3573</td>
<td>2624 ± 2163</td>
<td>432 ± 638</td>
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Anti-inflammatory cytokine IL-10 was weakly a little bit induced by GAS, LGG and Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892). The results are shown in Table 4 and Figure 4.

Compared with Lactococcus lactis ssp. cremoris ARH74 (DSM 18891), Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892) was more potent inducer of all pro-inflammatory and Th1 type cytokines.
**Table 4.** Dose-dependent IFN-γ production (pg/ml). Bacteria:host cell ratio of 0.2:1, 1:1 or 5:1 was used. Cell culture supernatants from bacteria stimulated supernatants were collected and cytokine levels measured with ELISA. Results are mean ± SD from four different blood donors.

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<td>PIA2</td>
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**Example 2. mRNA expression of cytokines**

The best inducers of pro- and anti-inflammatory cytokines were selected for the mRNA expression experiment.

Bacterial strains were grown, cell culture prepared and stimulation experiment carried out as described in example 1.

**RNA isolation and Northern Blotting**

Stimulated cells were collected, washed with PBS, and lysed in guanidium isothiocyanate, followed by a centrifugation through a CsCl cushion. Samples containing 10 μg total cellular RNA were size-fractionated on 1% formaldehyde-agarose gels, transferred to Hybond-N nylon membranes and hybridized at 42°C in a solution containing 50% formamide, 5 x Denhardt's solution, 5 x subacute sclerosing panencephalitis and 0.5% sodium dodecyl sulphate. Ethidium bromide staining was used for control equal loading.

**Results**

The kinetics of TNF-α mRNA expression was fast and it was clearly detectable already at 3 h after bacterial stimulation. However, p40, IFN-γ and IL-10 genes were induced with clearly slower kinetics and the expression of these mRNAs was detectable starting from 9 h after stimulation. LGG enhanced TNF-α mRNA expression already at 3 h. p40 and IL-10 mRNA expression was detectable at 9 h, but IFN-γ mRNA expression was seen at 24 h.

*Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) induced IFN-γ and IL-10 mRNA expression at 9 h. The strongest expressions
were detected in TNF-α and IFN-γ genes when stimulated with *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892).

**Example 3. Fermented milk product**

Standardised (3% fat content), homogenised, and heat-treated (95°C for 5 min) milk was cooled to 17-23°C. Milk was inoculated with a starter culture (L-type; consisting of *Lactococcus lactis* biovar *longi* and *Leuconostoc mesenteroides* ssp. *cremoris*) (1%) together with *Lactococcus lactis* subs. *cremoris* ARH74 (DSM 18891) (0.5%) and *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) (0.5%). Milk was packed and incubated for 20 h (until pH was below 4.6). Fermented milk product was cooled to a temperature below 6°C. It has mild and slightly acidic taste, high viscosity and ropy consistence.

Milk for a reference fermented milk product (i.e., a product without probiotic characteristics) was inoculated with a starter culture (L-type) (2%), consisting of *Lactococcus lactis* biovar *longi* and *Leuconostoc mesenteroides* ssp. *cremoris*.

**Example 4. Fermented milk product**

Standardised (3% fat content), homogenised, and heat-treated (95°C for 5 min) milk was cooled to 17-24°C. Milk was fermented a starter culture (DL-type/L-type; consisting of *Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp *cremoris* and *L. lactis* ssp. *lactis* biovar. *diacetylactis*) (1%) together with *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 (DSM 18892) (0.5%) in a tank for 17-24 h (until pH after cooling was below 4.3). The coagulum was broken gently, cooled to a temperature below 6°C. It has mild, slightly acidic and aromatic taste due to diacetyl and carbon dioxide produced from citrate metabolism of *Leuconostoc*.

Milk for a conventional fermented milk product (i.e., a product without probiotic characteristics) was inoculated with a starter culture (1.5%, DL-type, consisting of *Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp *cremoris* and *L. lactis* ssp. *lactis* biovar. *diacetylactis*).

**Example 5. Fermented milk product**

Standardised (3.5% fat content) (non-homogenised), and heat-treated (95°C for 5 min) milk was cooled to 20°C. Milk was inoculated with a starter culture (L-type; consisting of *Lactococcus lactis* ssp. *cremoris* biovar
longum and Leuconostoc mesenteroides ssp. cremoris (3%) together with Lactococcus lactis subs. cremoris ARH74 (DSM 18891) (0.5%) and Leuconostoc mesenteroides ssp. cremoris PIA2 (DSM 18892) (0.5%). A mould, Geotrichum candidum was added with the starter culture. The inoculated milk was packaged, placed on the trays and transferred to a ripening tunnel. The containers were incubated at 20°C for 18-24 h (until pH was below 4.6). Fermented milk product was cooled to a temperature below 6°C. It has mild and aromatic taste. The consistency is thick and slightly ropy but easily spoonable.

Milk for reference fermented milk product (without probiotic characteristics) was inoculated with a starter culture (L-type, consisting of Lactococcus lactis ssp. cremoris biovar longum and Leuconostoc mesenteroides ssp. cremoris).
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1-3-1: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

1-3-2: Inhoffenstr. 7B, D-38124 Braunschweig, Germany

1-3-3: 19 December 2006 (19.12.2006)

1-3-4: DSMZ 18891

2-3-1: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

2-3-2: Inhoffenstr. 7B, D-38124 Braunschweig, Germany


2-3-4: DSMZ 18892

2-4: According to Rule 13bis PCT the Applicant wishes to make use of the expert provisions in those countries which provide for such

2-5: all designations
| **0-4** | This form was received with the international application: (yes or no) | **YES** |
| **0-4-1** | Authorized officer | [Signature] |

**FOR INTERNATIONAL BUREAU USE ONLY**

| **0-5** | This form was received by the international Bureau on: |
| **0-5-1** | Authorized officer |
CLAIMS

1. A probiotic composition comprising *Leuconostoc* wherein the *Leuconostoc* is *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892.

2. A probiotic composition comprising *Leuconostoc* and *Lactococcus* wherein the *Lactococcus* is *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891.

3. The composition of claim 1 wherein the composition further comprises *Lactococcus*.

4. The composition of claim 3, wherein the *Lactococcus* is *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891.

5. The composition of claim 1 or claim 2, having a probiotic effect selected from immunomodulation, pro-inflammatory Th1 type immune response enhancing activity, anti-allergic activity, anti-atopic activity, anti-infectious activity and antiviral activity.

6. The composition of any one of claims 1 to 5, wherein it is for oral administration.

7. The composition of claim 1 or claim 2, wherein the bacteria is in a form selected from a live bacterial population, a lyophilized bacterial population, a fermented dairy product and a non-viable bacterial preparation.

8. The composition of claim 7, wherein said non-viable bacterial preparation is selected from heat-killed bacteria, irradiated bacteria and lysed bacteria.

9. The composition of any one of claims 1 to 8, wherein it further comprises conventional starter microbes and/or a prebiotic.

10. Use of the composition of any one of claims 1 to 9 in food industry, pharmaceutical industry, or in the manufacture of health promoting products or natural products.

11. Use according to claim 10, characterized in that the composition is added to a dairy product, drink, juice, soup or children's food.

12. Use of the composition of claim 1 or claim 2 for enhancing pro-inflammatory Th1 type immune response and/or for preventing or treating allergic, atopic, viral and/or infectious disease.

13. Use of the composition of claim 1 or claim 2 in the preparation of a product enhancing pro-inflammatory Th1 type immune response and/or for preventing or treating allergic, atopic, viral and/or infectious disease.
14. Use of *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 for enhancing pro-inflammatory Th1 type immune response and/or for the prevention or treatment of allergic, atopic, viral and/or infectious disease.

15. Use of *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 for enhancing pro-inflammatory Th1 type immune response and/or for the prevention or treatment of allergic, atopic, viral and/or infectious disease.

16. A method for protecting a subject against allergy and/or atopic disease and/or viral disease and/or infectious disease autoimmune diseases comprising the step of administering to said subject an effective amount of the probiotic composition of claim 1 or claim 2.

17. Use of a product obtained from fermenting milk by *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 for the prevention or treatment of allergy and/or atopic disease and/or viral disease and/or infectious disease.

18. The use of claim 17 wherein the product is obtained from fermenting milk by *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892 and *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891 for the prevention or treatment of allergy and/or atopic disease and/or viral disease and/or infectious disease.

Figure 1.

Figure 2.
Figure 3.

Figure 4.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC8: A61K, A23C, A23L, C12N

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

FI, SE, NO, DK

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

EPO-Internal, WPI, BIOSIS, MEDLINE, CAPLUS, FROSTI, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[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:

29 October 2008 (29.10.2008)

Date of mailing of the international search report:

05 November 2008 (05.11.2008)

Name and mailing address of the ISA/FI:

National Board of Patents and Registration of Finland
P.O. Box 1160, FI-00101 HELSINKI, Finland

Authorized officer

Kaarina Aarnisalo

Facsimile No. +358 9 6939 5328

Telephone No. +358 9 6939 500

Form PCT/ISA/210 (second sheet) (July 2008)
### 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. **X** Claims Nos.: **12, 14-18**
   
   because they relate to subject matter not required to be searched by this Authority, namely:

   Use of a composition for treatment of the human or animal body by therapy (Rule 39.1 (iv) PCT).

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

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

### 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:

See Extra Sheet

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

2. **X** As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

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

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

### Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.
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**CLASSIFICATION OF SUBJECT MATTER**

Int.Cl.

**A61K 35/74** (2006.01)
**A23C 9/123** (2006.01)
**A23L 1/30** (2006.01)
**A23L 1/03** (2006.01)
**C12N 1/20** (2006.01)
The application lacks unity within the meaning of Rule 13.1 PCT. According to Rule 13.1 PCT, an application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept.

Further, according to rule 13.2 PCT, the requirement of unity of invention is fulfilled only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" means those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The feature common to compositions of claims 1 and 2 is *Leuconostoc* bacteria having probiotic characteristics. This feature is known from the prior art, see for example JP 2007117031, JP 2006076961, EP 1082964 or JP 2007070249. Thus, this feature can not be considered to form a single inventive concept in the sense of Rule 13.1 PCT and Rule 13.2 PCT.

Consequently, the international application includes the following two inventions:

Invention I: Claims 1, 3 and 4; and claims 5-11, 13 when referring to claim 1; and claim 19

The claimed invention relates to the probiotic composition comprising *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892; to the use of the composition in food industry, pharmaceutical industry, or in the manufacture of health promoting products or natural products; and to the use of the composition in the preparation of a product enhancing pro-inflammatory Th1 type immune response and/or for preventing or treating allergic, atopic, viral and/or infectious diseases. The claimed invention further relates to the bacterial strain *Leuconostoc mesenteroides* ssp. *cremoris* PIA2, DSM 18892.

Invention II: Claims 1, 3 and 4; and claims 5-11, 13 when referring to claim 2

The claimed invention relates to the probiotic composition comprising *Leuconostoc* and *Lactococcus lactis* ssp. *cremoris* ARH74, DSM 18891; to the use of the composition in food industry, pharmaceutical industry, or in the manufacture of health promoting products or natural products; and to the use of the composition in the preparation of a product enhancing pro-inflammatory Th1 type immune response and/or for preventing or treating allergic, atopic, viral and/or infectious diseases.