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(54) Title: IMPROVED IMMUNOMODULATION BY PROBIOTICS

(57) Abstract: The invention relates to a method for preparing a bacterium capable of immunomodulation, having either anti-in-
flammatory or pro -inflammatory capacities, by incorporating or knocking-out one or more genes. The invention also relates to
modulating expression of these one or more genes by adapting fermentation conditions. The invention further provides for a
method for identifying a bacterium capable of immunomodulation, a recombinant bacterium obtainable by any of such methods,
compositions comprising such recombinant bacterium, and their use as a medicament. Also, the use of protein and nucleic acid se-
quences as a medicament is described.



Improved immunomodulation by probiotics

Field of the invention

The invention relates to a method for identifying a bacterium capable of immunomodulation, a method for preparing a recombinant bacterium capable of immunomodulation and a recombinant bacterium obtainable by such method, a composition comprising such recombinant bacterium, including its use as a medicament, in particular for prevention and/or treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis. Also, the invention relates to a method for modulating certain genes involved in immunomodulatory capacity. Moreover, the invention relates to the use of certain proteins and nucleic acid constructs as a medicament.

Background

Inflammatory bowel disease ("IBD") refers to a group of gastrointestinal ("GI") disorders characterised by a chronic nonspecific inflammation of portions of the GI tract. Crohn's disease and ulcerative colitis are the most prominent examples of IBD.

Ulcerative colitis refers to a chronic, nonspecific, inflammatory, and ulcerative disease having manifestations primarily in the colonic mucosa. It is often characterised by bloody diarrhoea, abdominal cramps, blood and mucus in the stools, malaise, fever, anaemia, anorexia, weight loss, leukocytosis, hypoalbuminemia, and an elevated erythrocyte sedimentation rate (ESR). Complications include haemorrhage, toxic colitis, toxic megacolon, occasional rectovaginal fistulas, and an increased risk for the development of colon cancer.

Crohn's disease shares many features with ulcerative colitis. It is distinguishable in that lesions tend to be sharply demarcated from adjacent normal bowel, in contrast to the lesions of ulcerative colitis which are fairly diffuse.

Treatment is similar for both diseases. It includes administration of steroids, sulphasalazine and its derivatives, and immunosuppressive drugs such as cyclosporine A, mercaptopurine, and azathiopurine.

The cause of IBD is unknown. The pathogenesis probably involves interaction between genetic and environmental factors, although no definite etiological agent has

been identified so far. The main theory is that abnormal immune response, possibly driven by intestinal microflora, occurs in IBD. It is well established that T cells play an important role in pathogenesis. Activated T cells can produce both anti-inflammatory and pro-inflammatory cytokines.

5 Interleukin (IL)-10 is known as a major endogenous anti-inflammatory intercessor and can be produced by most of the body's immune cells. It controls and suppresses inflammation essentially by down-regulating pro-inflammatory cytokine production, most likely by its NF- κ B blocking activity, and class II antigen presentation. As such, IL-10 can counteract inflammation through its activity on
10 antigen presenting cells, which in turn affect T cell activity. IL-10 can, however, also directly suppress T cell proliferation. Polymorphonuclear leukocytes are an important source of pro-inflammatory cytokines in patients with intestinal inflammation and can also be down-regulated by IL-10.

In contrast, Interleukin (IL)-12 is a multifunctional pro-inflammatory cytokine. It
15 activates NK and T cells to produce several cytokines, especially INF- γ . In addition, it enhances the cytotoxic activation of activated NK cells and favors the generation of cytolytic T cells. IL-12 also enhances the phagocytic and bacteriocidal activity of phagocytic cells and their ability to release pro-inflammatory cytokines, including itself. IL-12 is the key immunoregulator molecule favoring the differentiation and
20 function of Th1 cells and inhibiting the differentiation of Th2 cells.

IL-10 and IL-12 have opposite roles in the regulation of the cytokine network, and as such they have attracted interest in various clinical instances, such as autoimmune diseases, allograft rejection, arthritis, atherosclerosis, gynecology and ophthalmology. The upregulation of IL-10 compared to IL-12 is of interest to
25 producing an anti-inflammatory response, such as for prevention and/or treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis, and/or for stimulation of the immune system, e.g. in the case of hay fever. In contrast, the upregulation of IL-12 compared to IL-10 may be interesting to produce a pro-inflammatory response, e.g., for use in vaccination purposes, such as for use as an
30 adjuvant for a vaccine.

US 5,368,854 discloses a method for treating IBD by parenteral administration of IL-10. However, such an administration route has several drawbacks. Oral administration of IL-10 would provide a much easier and more convenient way, and

localized release of IL-10 allows for higher efficacy and less unwanted side effects due to systemic activities. However, IL-10 is highly acid-sensitive and would as such not survive passage through the GI tract. As such, one needs to find a way to effectively administer IL-10 in the GI tract.

5 US 2002/0019043 describes administering IL-10 using recombinant *Lactococcus lactis* cells that are engineered to produce IL-10 *in situ* by incorporation of a heterologous gene. In principle, *L. lactis* is a foodgrade bacterium, however, recombinant microorganisms are presently not regarded as safe, and are as such not allowed in food products.

10 It is known that probiotics may have immunomodulatory properties (see, e.g., WO 2008/079009, JP2008-099632, WO 2007/040446, US 2007/0148148, and the like). However, at present it is unknown which bacterial factors and/or cellular receptors contribute to the immunomodulatory properties of various probiotic bacteria. In the present study 42 different *Lactobacillus plantarum* strains were tested for their
15 capacity to stimulate PBMC and/or dendritic cells. As IL-10 and IL-12 are oppositely acting cytokines, the IL-10 over IL-12 ratios were determined to establish immunomodulatory properties of these strains, a high IL-10/IL-12 ratio indicating anti-inflammatory capacity, and a low IL-10/IL-12 ratio indicating pro-inflammatory properties (Foligne *et al.* World J. of Gastroenterol. 2007. Vol. 13(2):236-243). The
20 immunoprofiling results were correlated with a *L. plantarum* WCFS1-based genome-wide genotype database. This led to the identification of genes encoding novel candidate immuno-effector compounds. Knowledge of the specific probiotic cell components influencing host responses will result in the rational selection and cultivation of probiotic strains for specific health benefits.

25

Summary of the Invention

In a first aspect, the present invention relates to a method for preparing a bacterium capable of immunomodulation, said method comprising the step of knocking
30 out or incorporating one or more genes encoding: a) a polypeptide of a N-acetyl-galactosamine phosphotransferase system; b) a polypeptide encoded by a *lamBDCA* gene cluster; c) a polypeptide encoded by a bacteriocin gene cluster; d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by

SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

In an embodiment, said bacterium has anti-inflammatory capacities, and said method comprises the step of knocking out one or more genes encoding: i) a polypeptide of a N-acetylgalactosamine phosphotransferase system; ii) a polypeptide encoded by a *lamBDCA* gene cluster; iii) a polypeptide encoded by a bacteriocin gene cluster; iv) a polypeptide encoded by a bacteriocin transport gene cluster; and/or v) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

In another embodiment, said bacterium has pro-inflammatory capacities, said method comprising the step of incorporating one or more genes encoding: i) a polypeptide of a N-acetylgalactosamine phosphotransferase system; ii) a polypeptide encoded by a *lamBDCA* gene cluster; iii) a polypeptide encoded by a bacteriocin gene cluster; iv) a polypeptide encoded by a bacteriocin transport gene cluster; and/or v) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

In a further aspect, the present invention relates to a method for modulating expression of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster, and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, said method comprising the step of selecting fermentation conditions resulting in reduced or increased expression of one or more polypeptides according to any one of a), b) and c) compared to standard fermentation conditions.

In yet another aspect, the invention pertains to a method for identifying a bacterium capable of immunomodulation, said method comprising the step of detecting the presence or absence of expression of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster, and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith.

The polypeptide of a N-acetylgalactosamine phosphotransferase system may be selected from SEQ ID NO.s 1, 2, 3, 4 or 5, or homologues thereof having at least 25% identity therewith.

5 The polypeptide encoded by a *lamBDCA* gene cluster may be selected from SEQ ID NO.s 16, 17, 18, or 19, or homologues thereof having at least 25% identity therewith.

The polypeptide encoded by a bacteriocin gene cluster may be selected from SEQ ID Nos 6, 7, 8 or 9, or homologues thereof having at least 25% identity therewith.

10 The polypeptide encoded by a bacteriocin transport gene cluster may be selected from SEQ ID Nos:10, 11, 12, 13, 14, and/or 15, or homologues thereof having at least 25% identity therewith.

In an embodiment, the bacterium is a Gram-positive bacterium, preferably a lactic acid bacterium, more preferably belonging to the genus *Lactobacillus*.

15 The invention is also directed to a bacterium obtainable by the methods of the present invention, as well as a composition comprising such bacterium and a pharmaceutically or physiologically acceptable carrier.

In another aspect, the present invention provides for a bacterium or a composition as above, or a bacterium lacking a polypeptide of a N-acetylgalactosamine phosphotransferase system, lacking a polypeptide encoded by a *lamBDCA* gene cluster, lacking a polypeptide encoded by a bacteriocin gene cluster, lacking a polypeptide encoded by a bacteriocin transport gene cluster, and/or lacking a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, as defined herein for use as a medicament, in particular for treatment of inflammatory bowel disease such as Crohn's disease and ulcerative colitis.

25 Another aspect of the invention is concerned with a polypeptide of a N-acetylgalactosamine phosphotransferase system, a polypeptide encoded by a *lamBDCA* gene cluster, or a polypeptide encoded by a bacteriocin gene cluster, for use as a medicament, in particular for prevention and/or treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis, and/or for stimulation of the immune system, e.g., as a vaccine adjuvant.

30 The invention also relates to a nucleic acid construct comprising one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide

encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster, and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, operably linked to a regulating region, for use as a medicament, in particular for prevention and/or treatment
5 of inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and/or for stimulation of the immune system, e.g. as a vaccine adjuvant.

Detailed description of the invention

The present inventors have tested 42 different *Lactobacillus plantarum* strains for
10 their capacity to stimulate PBMC (determination of IL-10 and IL-12 secretion), and also for their capacity to stimulate dendritic cells (DCs). As IL-10 and IL-12 are oppositely acting cytokines, the IL-10 over IL-12 ratios were determined to establish immunomodulatory properties of these strains, a high IL-10/IL-12 ratio indicating anti-inflammatory capacity, and a low IL-10/IL-12 ratio indicating pro-inflammatory
15 properties. It was found that the *L. plantarum* strains varied in their ability to stimulate the secretion of IL-10 and IL-12

The immunoprofiling results were subsequently correlated with a *L. plantarum* WCFS1-based genome-wide genotype database. *L. plantarum* is a common inhabitant of the human GI tract. *L. plantarum* WCFS1 is a single colony isolate of the esophageal
20 *L. plantarum* strain NCIMB8826, which was shown to survive stomach passage in an active form (Vesa *et al.* Aliment. Pharmacol. Ther. 2000 Jun;14(6):823-8). Its genome has been sequenced and appears to be one of the largest genomes known among lactic acid bacteria (3.3 Mb).

Correlation of the IL-10 levels elicited by the *L. plantarum* strains to gene
25 presence/absences scores against the *L. plantarum* WCFS1 genome resulted in the identification of 11 genes with putative roles in IL-10 stimulation. These 11 genes were divided in four clusters: strains containing homologs of lp_1953 (table 3) showed, on average, a 1.6-fold higher IL-10 stimulating capacity compared to strains without these genes. Strains containing homologs of the multi-gene locus lp_2647 to lp_2651
30 induced, on average, a 1.7x lower IL-10 stimulating capacity compared to strains without these genes. Lp_2647 to lp_2651 encode Pts19ADCBR, a *N*-acetyl-galactosamine phosphotransferase system and putative transcription regulator. Homologs of this operon are present in 33% of the tested strains. Another gene,

lp_2991, is annotated as a transcription regulator which is present in 90% of the strains tested. Finally, four of these genes lie within the multigene locus (lp_0423 to lp_0429) involved in plantaracin biosynthesis and secretion.

Correlations between CGH data and IL-12 levels among the *L. plantarum* strains did not result in identification of specific genes which might modulate expression levels of this cytokine in PBMCs or DCs. The Random Forests method returned no genes with high variable important measures, indicating that other factors than presence/absence of the genes in the WCFS1 genome are responsible for the observed variation between bacterial strains.

Comparisons between the CGH data and IL-10/IL-12 ratios induced by the *L. plantarum* strains, resulted in the identification of 7 genes for which the presence/absence profile in the bacterial strains correlated with the IL-10/IL-12 ratio. Lp_0419 to lp_0423 (SEQ ID Nos: 6-9) and lp_3582 (SEQ ID NO:13) were predominantly present in strains stimulating a low IL10/IL12 ratio. Lp_0419 to lp_0422 are the *plnEFI* operon, encoding two bacteriocin-like peptides and a bacteriocin immunity protein and homologs of the genes in this operon are present in 81-85% of the tested strains. Lp_0423 is distal to lp_0422, located in another operon, and encodes an ABC transporter involved in the transport of bacteriocins (Diep, D. B., L. S. Havarstein, and I. F. Nes. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol 178:4472-4483). Lp_3582 encodes an accessory gene regulator protein B (LamB) of the *lamBDCA* operon (SEQ ID NO: 10-13). This operon encodes for the *Lactobacillus* agr-like quorum sensing module important for biofilm formation and regulation of adherence (Sturme, M. H., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, T. Fujii, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos. 2005. An agr-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J Bacteriol 187:5224-5235). A homolog of lp_3582 is present in 33% of the tested strains.

The involvement of the candidate genes/gene clusters mentioned above in cytokine secretion was validated using specific deletion mutants. It was found that deletion mutants lacking genes involved in plantaracin (bacteriocin) secretion and immunity induced significantly higher amounts of IL-10 and higher ratios of IL-10/IL-12 in both DCs and PBMCs compared to the wild type strain WCFS1. Deletion of the

bacteriocin transport operon also significantly induced IL-10. Deletion of lp_2991 was associated with induction of IL-10 and TNF- α (and IL-10/IL-12 ratio). Similarly, deletion of Lp_2647 to lp_2651 encoding Pts19ADCBR, the N-acetylglucosamine phosphotransferase system and putative transcription regulator, induced IL-10 (and
5 increased IL-10/IL-12 ratio) in PBMCs compared to the wild type strain WCFS1. A $\Delta lamAR$ deletion mutant also induced higher IL-10/IL-12 ratios than the wild type WCFS1 strain.

Bacteriocins are secreted oligopeptides, proteins or protein complexes with antimicrobial activity against bacteria closely related to the producer organism. It is
10 well known that they are produced by gram-positive bacteria, including LAB. In *L. plantarum* WCFS1, *plnEFI* (lp_0419 to lp_0423) is the gene cluster encoding two different bacteriocins (PlnE, SEQ ID NO:8; and PlnF, SEQ ID NO:7), and an immunity protein PlnI (SEQ ID NO:6). Homologs of the gene loci in this operon are present in
15 81-85% of the tested strains. It is hypothesized that the two bacteriocins are required to achieve biological activity. The immune protein serves to protect the bacterium from its own bacteriocins (Diep et al. 1996, J. Bacteriol., vol. 178, pp 4472-4483).

The multigene locus lp_0423 to lp_0429 is involved in bacteriocin (plantaricin) biosynthesis and secretion. It encodes plnGHSTUVW, an ABC transporter system.

Lp_2991 is annotated as a transcription regulator which is present in 90% of the
20 strains tested.

The N-acetylgalactosamine phosphotransferase system (PTS) (lp_2647-lp_2651) is a structurally and functionally complex system for transporting sugars. It consists of substrate-recognizing protein constituents (Enzymes II). The sugar substrate is transported from the extracellular medium through the membrane in a pathway
25 determined by the integral membrane permease-like Enzyme IIC constituent, often a homodimer in the membrane. The sequentially-acting energy-coupling proteins transfer a phosphoryl group from the initial phosphoryl donor, phosphoenolpyruvate, to the ultimate phosphoryl acceptor, sugar, yielding a sugar-phosphate.

The *lamBDCA* operon (lp_3580 to lp_3582; SEQ ID NOS:10-13) encodes a two-
30 component regulatory system in *Lactobacillus plantarum* with homology to *agrBDCA* and *fsrABC* quorum sensing systems of *Staphylococcus aureus* and *Enterococcus faecalis*, respectively (Sturme et al. 2005, J. Bacteriol., vol. 187, no. 15, pp. 5224-5235). The *lamBDCA* genes are predicted to code for a histidine protein kinase LamC

(SEQ ID NO:11), its cognate response regulator LamA (SEQ ID NO:10), LamB (SEQ ID NO:13), and LamD (SEQ ID NO:12). LamD seems to be a precursor of a cyclic thiolactone auto-inducing peptide (a well-known signalling molecule in Gram-positive bacteria). Sturme *et al.* (*supra*) postulate that the *L. plantarum lamBDCA* system may
5 play a role in commensal host-microbe interactions.

The present invention relates to a method for preparing a bacterium capable of immunomodulation, said method comprising the step of knocking out or incorporating one or more genes encoding: a) a polypeptide of a N-acetyl-galactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a
10 polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

As set forth above, a high IL-10/IL-12 ratio correlates with the absence of a polypeptide of a N- acetyl galactosamine phosphotransferase system, and/or the absence
15 of a polypeptide encoded by a *lamBDCA* gene cluster, and/or the absence of a polypeptide encoded by a bacteriocin gene cluster, and/or the absence of a polypeptide encoded by a bacteriocin transport gene cluster, and/or the absence of a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith.

20 The terms “immunomodulation”, “immunomodulatory properties” and “immunomodulatory capacities” refer to a change in the body’s immune system, caused by agents that either activate or suppress its function. Immunomodulation may be either anti-inflammatory or pro-inflammatory. According to the present invention, anti-inflammatory capacities are characterized by a high IL-10 over IL-12 ratio, whereas
25 pro-inflammatory capacities are characterized by a low IL-10 over IL-12 ratio. As used herein, a “high IL-10 over IL-12” ratio refers to a ratio that is significantly increased ($p < 0.05$) compared to the IL-10 over IL-12 ratio of the wild type strain *L. plantarum* WCFS1 when tested under identical conditions. In an embodiment, the IL-10 over IL-12 ratio is increased by at least 10%, such as at least 20%, 30%, 40%, 50%, 60%, 70%,
30 80%, 90%, 100%, 125%, 150%, 175%, 200%, or more.

The bacterium may be any bacterium, in particular a Gram-positive bacterium. Preferably, the bacterium is a lactic acid bacterium (LAB), preferably a probiotic LAB. A “probiotic” refers to a live microorganism which when administered in adequate

amounts confer a health benefit on the host. Lactic acid bacteria (LAB) are the most common type of microbes used. LAB have been used in the food industry for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. This not only provides the characteristic sour taste of fermented dairy foods such as yogurt, but also by lowering the pH may create fewer opportunities for spoilage organisms to grow, hence creating possible health benefits on preventing gastrointestinal infections. Strains of the genera *Lactobacillus* and *Bifidobacterium*, are the most widely used probiotic bacteria. For example, the bacterium may belong to the genera *Lactobacillus*.

10 The bacterium is preferably a recombinant bacterium. The term “recombinant bacterium”, as used herein, refers to a bacterium whose genetic makeup has been altered by deliberate introduction of new genetic elements. Such recombinant bacterium may be prepared by methods well known in the art. E.g., one or more genes may be added to the bacterium’s genetic makeup, i.e., may be incorporated. Such incorporation of said one or more genes may be carried out using techniques well known in the art, such as using vectors. Alternatively, one or more genes may be knocked out, as further explained below. The term “recombinant bacterium” may also include so-called “clean deletion mutants”, i.e. deletion mutants that do not contain any foreign DNA. Such clean deletion mutants may be constructed using approaches involving suicide vectors such as pUC19. Procedures for obtaining clean deletion mutants have been described by Lambert et al. (Lambert JM, Bongers RS, Kleerebezem M. Appl Environ Microbiol. 2007 Feb;73(4):1126-35). Such (clean) deletion mutants may be distinguished from a naturally occurring bacterium using a PCR approach involving PCR primers in the flanking region of the mutagenised gene, as the resulting amplicon will be distinctly smaller for the mutant compared to the wild type strain.

25 The term “gene” means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked sequences, such as a promoter, a 5’ leader sequence comprising e.g. sequences involved in translation initiation, a (protein) coding region (cDNA or genomic DNA) and a 3’non-translated sequence comprising e.g. transcription termination sites.

A "vector" is herein understood to mean a man-made nucleic acid molecule resulting from the use of recombinant DNA technology and which is used to deliver DNA into said bacterium. The vector backbone may for example be a binary or superbinary vector, a co-integrate vector or a T-DNA vector, as known in the art and as described elsewhere herein, into which a (chimeric) gene may be integrated or, if a suitable transcription regulatory sequence is already present, only a desired nucleic acid sequence (e.g. a coding sequence, an antisense or an inverted repeat sequence) is integrated downstream of the transcription regulatory sequence. Vectors usually comprise further genetic elements to facilitate their use in molecular cloning, such as e.g. selectable markers, multiple cloning sites and the like. A "chimeric gene" (or recombinant gene) refers to any gene, which is not normally found in nature in a species, in particular a gene in which one or more parts of the nucleic acid sequence are present that are not associated with each other in nature. For example the promoter is not associated in nature with part or all of the transcribed region or with another regulatory region. The term "chimeric gene" is understood to include expression constructs in which a promoter or transcription regulatory sequence is operably linked to one or more coding sequences.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter, or rather a transcription regulatory sequence, is operably linked to a coding sequence if it affects the transcription of the coding sequence.

In an advantageous embodiment, the bacterium to be prepared is a recombinant bacterium, i.e. prepared using recombinant DNA technology. The one or more genes encoding the proteins of interest may be 'knocked out' or inactivated by one or more

of: deletion, insertion or mutation of the respective gene; replacing the promoter of the gene with a weaker promoter; antisense DNA or RNA; and siRNA. The knocking out or inactivation of the one or more genes encoding the proteins result in essentially non-functional proteins. The term “essentially non-functional proteins” as used herein means that the protein is not or only to a small extent capable of performing its natural function in the bacterium.

The amino acid sequence of may be altered to produce essentially non-functional protein(s). To this end, amino acid residues may be deleted, inserted or mutated, to yield a non-functional protein of interest. A mutation of the amino acid sequence is understood as an exchange of the naturally occurring amino acid at a desired position for another amino acid. Site-directed mutagenesis may be applied to, for example, alter amino acid residues in the catalytic site, amino acid residues that are important for substrate binding, cofactor binding, or binding to effector molecules, amino acid residues that are important for correct folding, or structurally important domains of the proteins. The amino acid sequence may be mutated using site-directed mutagenesis, or may alternatively be mutated using random mutagenesis, e.g., using UV irradiation, chemical mutagenesis methods or random PCR methods. Alternatively, the one or more genes may be partially or completely deleted or inactivated using well-known knock-out techniques. Another alternative is replacing the natural promoter of the gene with a weaker or inactive promoter, resulting in lack of expression of the protein in question. The skilled person knows how to replace the natural promoter with another promoter.

It is routine work for the skilled person to choose an adequate strategy to introduce a suitable modification of the gene(s) of interest in order not to get expression of a functional protein. For example, methods for *in vitro* mutagenesis are described in Sambrook et al. (Molecular cloning, A laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 1989). Corresponding methods are also available commercially in the form of kits (e.g., Quikchange site-directed mutagenesis kit by Stratagene, La Jolla, USA). Gene deletion may, for example, be accomplished by the gene replacement technology that is well known to the skilled person.

The amino acid sequences of the proteins of interest are publicly available in amino acid sequence databases. Homologues thereof in various bacteria can be easily identified using Blast searches, as is well known to the skilled person.

The gene encoding the protein(s) of interest may also be silenced (or “switched off”) using antisense DNA or (m)RNA or RNAi, preferably siRNA. The term gene silencing is generally used to describe the switching off of a gene by a mechanism other than genetic modification. That is, a gene which would be expressed under normal circumstances is switched off by machinery in the cell. The skilled person knows how to apply gene silencing to the present invention, and how to select and prepare a suitable gene silencing construct.

In an embodiment, ‘knock-out’ or ‘deletion’ mutants can be constructed that do not contain any foreign DNA, a so called clean deletion mutant, e.g., using suicide vectors such as pUC19 (Lambert JM, et al. Appl Environ Microbiol. 2007. 73(4):1126-1135). Although more laborious to prepare, such clean deletion mutants do not comprise any heterologous DNA and as such may more readily be used in the preparation of food products. Distinction of (clean) deletion mutants and the natural bacterium can be done by a PCR approach using primers in the flanking regions of the mutagenised gene, as the resulting amplicon will be distinctly smaller for the mutant as compared to the wild-type.

Alternatively, the one or more gene(s) encoding a) a polypeptide of a N-acetyl-galactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, may be incorporated in the bacterium, preferably in a manner leading to stable expression. The person skilled in the art is aware of method for stably incorporating genes encoding a polypeptide of interest into a bacterium.

The skilled person will understand that the polypeptides as defined in the invention could be present in other bacteria than from the herein specified *Lactobacillus plantarum* strain, i.e., they could be homologues thereof, e.g., from other *Lactobacilli* species or from other probiotic species as long as it has the identity and functionality defined herein. A preferred bacterium is a food-grade bacterium, or a commensal bacterium.

A polypeptide of a N-acetylgalactosamine phosphotransferase system may be any polypeptide selected from Enzyme IIA, Enzyme IIB, Enzyme IIC, Enzyme IID and the transcription regulator. In an embodiment, the polypeptide is selected from an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 2, 3, 4 or 5, or
5 homologues thereof having at least 25% identity therewith. Homologues of SEQ ID NOS:2, 3, 4, 5 or 6 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:1, 2, 3, 4, or 5, respectively.

A polypeptide encoded by a *lamBDCA* gene cluster may be any polypeptide
10 selected from histidine protein kinase LamC, response regulator LamA, LamB, and LamD. In an embodiment, the polypeptide is selected from an amino acid sequence selected from the group consisting of SEQ ID NOS: 16, 17, 18, and 19 (LamA, LamC, LamD and LamB, respectively), or homologues thereof. Homologues of SEQ ID NOS:10, 11, 12, or 13 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%,
15 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:16, 17, 18, and 19, respectively. A polypeptide encoded by a bacteriocin gene cluster may be any polypeptide selected from bacteriocins PlnE (SEQ ID NO:8) and PlnF (SEQ ID NO:7), and an immunity protein PlnI (SEQ ID NO:6) or homologues thereof having at least 25% identity therewith.
20 Homologues of SEQ ID NOS:6, 7, or 8 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:6, 7, or 8, respectively.

A polypeptide encoded by a bacteriocin transport gene cluster may be any polypeptide selected from bacteriocin ABC-transporter, ATP-binding and permease
25 protein PlnG (SEQ ID NO:9), bacteriocin ABC-transporter, accessory factor PlnH (SEQ ID NO:10), plantaricin biosynthesis protein PlnS (SEQ ID NO:11), and integral membrane proteins PlnT, PlnU, PlnV, and PlnW (SEQ ID NOS:12, 13, 14, and 15, respectively), or homologues thereof having at least 25% identity therewith. Homologues of SEQ ID NOS:9, 10, 11, 12, 13, 14, or 15 preferably have at least 25%,
30 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:9, 10, 11, 12, 13, 14, or 15, respectively.

Homologues of SEQ ID NO:20 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NO:20.

As used herein, percentage of identity is calculated as the number of identical amino acid residues between aligned sequences divided by the length of the aligned sequences minus the length of all the gaps. Sequence alignment may e.g. be performed using DNAMAN 4.0 optimal alignment program using default settings. One skilled in the art is well aware of how to determine the percentage of identity.

A recombinant bacterium having anti-inflammatory capacities may be prepared by knocking out one or more genes encoding: i) a polypeptide of a N-acetylgalactosamine phosphotransferase system; ii) a polypeptide encoded by a *lamBDCA* gene cluster; iii) a polypeptide encoded by a bacteriocin gene cluster, iv) a polypeptide encoded by a bacteriocin transport gene cluster; and/or v) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

In an embodiment, said recombinant bacterium carries knock-outs in two, three, four or more, preferably in all five of the N-acetylgalactosamine phosphotransferase system, *lamBDCA* gene cluster, or bacteriocin gene cluster, bacteriocin transport gene cluster, and SEQ ID NO:20 or homologues thereof, yielding non-working systems. Knocking out a single gene in said one or more gene clusters encoding polypeptides which are part of the N-acetylgalactosamine phosphotransferase system, *lamBDCA* system, bacteriocin system, or bacteriocin transport system, may suffice to yield a non-functional N-acetylgalactosamine phosphotransferase system, *lamBDCA* system, bacteriocin system, or bacteriocin transport system.

A recombinant bacterium having pro-inflammatory capacities may be prepared using a method comprising the step of incorporating one or more genes encoding: i) a polypeptide of a N-acetylgalactosamine phosphotransferase system; ii) a polypeptide encoded by a *lamBDCA* gene cluster; iii) a polypeptide encoded by a bacteriocin gene cluster, iv) a polypeptide encoded by a bacteriocin transport gene cluster; and/or v) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in said bacterium.

In an embodiment, in said recombinant bacterium preferably all genes encoding polypeptides of one of i) a N-acetylgalactosamine phosphotransferase system; ii) a

lamBDCA gene cluster; iii) a bacteriocin gene cluster, iv) a polypeptide encoded by a bacteriocin transport gene cluster; and v) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, are incorporated, yielding either a functional N-acetylgalactosamine phosphotransferase system, a functional *lamBDCA* system, a functional bacteriocin system, a functional bacteriocin transport system, and/or a functional transcription regulator having the amino acid sequence of SEQ ID NO:20 or a functional homologue thereof. Preferably, genes are incorporated encoding all polypeptides to yield two or more, and preferably three or more, such as four or all five, of a functional N-acetylgalactosamine phosphotransferase system, a functional *lamBDCA* system, a functional bacteriocin system, a functional bacteriocin transport system, and a functional transcription regulator having the amino acid sequence of SEQ ID NO:20 or a functional homologue thereof. A bacterium having a functional N-acetylgalactosamine phosphotransferase system is characterized by its ability to transport N-acetylgalactosamine into said bacterium.

15 A functional *lamBDCA* system is a system resulting in production of a cyclic thiolactone auto-inducing peptide.

A functional bacteriocin system is characterized by production of at least one, and preferably two, bacteriocins.

20 A functional bacteriocin transport system is characterized by its ability to transport bacteriocins.

In a further aspect, the present invention pertains to a method for modulating expression of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system; b) a polypeptide encoded by a *lamBDCA* gene cluster; c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, in a bacterium, said method comprising the step of selecting fermentation conditions resulting in increased or reduced expression of one or more polypeptides according to any one of a), b) and c) compared to standard fermentation conditions.

30 Fermentation conditions may be varied to optimize expression levels of the effector molecules mentioned above, for example by varying medium composition at the level of osmolality, nutrient availability, and/or by changing growth conditions, level of available oxygen, pH, temperature, and the like. The skilled person knows how

to optimize medium composition to modulate expression of the genes referred to above. For example, fermentation conditions may be optimized by varying the salt concentration (e.g., from 0 to 0.3 M), by performing the fermentation either under aerobic or anaerobic conditions, by varying the amino acid concentration in the medium, and the like. Transcriptomics data of all fermentation conditions may be collected, and based on these data, fermentation conditions may be selected in which the effector molecules referred to hereinabove are expressed at a specific level that will lead to modulation of IL-10/IL-12 ratio. In an advantageous embodiment, chemically defined medium (CDM; Poolman and Konings. 1988. J. Bacteriol. 170(2):700-707).

As used herein, the term "standard fermentation conditions" refers to fermentation of a bacterium in 2 x CDM (chemically defined medium) medium, under anaerobic conditions, without addition of salt or any other additives, at 37°C, at pH 5.8.

The temperature may be varied at any temperature such as between 25 and 42°C. The pH may be varied at any pH such as at a pH of between 4 and 8. Suitable pH steps may include pH 5.2, 5.8, 6.5, and so on.

The method of the invention allows assessment of (industrial) fermentation processes for the expression levels of the effector molecules as described above influencing IL-12, IL-10, TNF- α by e.g. quantitative RT-PCR which may predict to what extent probiotic strains are capable of immunomodulation.

The invention also provides for a method for identifying a bacterium capable of immunomodulation, said method comprising the step of detecting the presence or absence of expression of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system, b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith.

In a first step, the presence or absence of the one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system; b) a polypeptide encoded by a *lamBDCA* gene cluster; c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, may be established. One skilled in the art is aware of methods for

establishing the presence or absence of such genes. Genomic DNA of bacteria may be isolated by methods well known in the art, such as those described in Sambrook *et al.* (Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; 3 Lab edition (January 15, 2001)). Also, kits for extraction of genomic DNA are commercially available from Qiagen, Favorgen, and the like. The genomic DNA may subsequently be probed for presence or absence of the gene in question using any well-known method in the art, e.g. Polymerase Chain Reaction selectively amplifying the gene(s) in question, or a simple hybridization assay using a probe selectively hybridizing to the gene(s) in question. The absence of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system; b) a polypeptide encoded by a lamBDCA gene cluster; c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, is indicative of a bacterium having anti-inflammatory capacities. In contrast, the presence of one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system; b) a polypeptide encoded by a lamBDCA gene cluster; c) a polypeptide encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, is indicative of a bacterium having pro-inflammatory capacities.

Once the presence of the one or more genes has been established, in a following step the expression of the genes may be assessed. "Expression of a gene" refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide (or active peptide fragment) or which is active itself (e.g. in posttranscriptional gene silencing or RNAi). An active protein in certain embodiments refers to a protein being constitutively active. The coding sequence is preferably in sense-orientation and encodes a desired, biologically active protein or peptide, or an active peptide fragment.

Establishing expression of a gene may be carried out by isolating RNA, in particular mRNA, from the bacteria. The absence of mRNA encoding the polypeptides of either a), b), c), d) and/or e) is indicative of a bacterium having anti-inflammatory

capacities. In contrast, the presence of mRNA encoding the polypeptides of either a), b), c), d) and/or e) is indicative of a bacterium having pro-inflammatory capacities.

The polypeptide of a N-acetyl-galactosamine phosphotransferase system is preferably selected from SEQ ID Nos: 1, 2, 3, 4, or 5, or homologues thereof having at least 25% identity therewith.

The term "homologues" refers to molecules in other bacteria, or variants prepared by recombinant DNA technology performing essentially the same function as the protein of interest.

In an embodiment, the polypeptide encoded by a lamBDCA gene cluster is selected from SEQ ID Nos: 16, 17, 18, or 19, or homologues thereof having at least 25% identity therewith.

In an embodiment, the polypeptide encoded by a bacteriocin gene cluster is selected from SEQ ID Nos: 6, 7, or 8, or homologues thereof having at least 25% identity therewith.

In an embodiment, the polypeptide encoded by a bacteriocin transport gene cluster is selected from SEQ ID Nos: 9, 10, 11, 12, 13, 14, or 15, or homologues thereof having at least 25% identity therewith.

Also encompassed by the present invention is a recombinant bacterium obtainable by the methods of the invention. In an embodiment, said recombinant bacterium is not a *lamA*-defective recombinant *Lactobacillus plantarum* WCFS1 mutant or a *lamBD*-overexpressing *Lactobacillus plantarum* WCFS1 mutant, both as disclosed in Sturme et al. (J. Bacteriol. 2005. Vol. 187, no. 5:5224-5235), or *L. plantarum* WCFS1 Δ *lamAR* (*lp_3580* and *lp_3087*) as disclosed by Fujii et al. (Fujii, T., C. Ingham, J. Nakayama, M. Beerthuyzen, R. Kunuki, D. Molenaar, M. Sturme, E. Vaughan, M. Kleebezem, and W. de Vos. 2008. Two Homologous agr-like Quorum Sensing Systems co-operatively Control Adherence, Cell morphology, and Cell Viability Properties in *Lactobacillus plantarum* WCFS1. J Bacteriol. 2008. 190(23):7655-65).

Moreover, the present invention is concerned with a composition comprising such recombinant bacterium and a pharmaceutically or physiologically acceptable carrier. Such composition may be a nutritional composition, such as a food composition. A recombinant bacterium according to the present invention may be cultured under appropriate conditions, optionally recovered from the culture medium

and optionally formulated into a composition suitable for the intended use. Methods for the preparation of such compositions are known *per se*.

A composition for oral administration may be either a food composition or a pharmaceutical composition. A pharmaceutical composition will usually comprise a pharmaceutical carrier in addition to said recombinant bacterium. A pharmaceutical carrier can be any compatible, nontoxic substance suitable to deliver said recombinant bacterium to the GI tract of a subject. For example, sterile water or inert solids may be used as a carrier usually complemented with a pharmaceutically acceptable adjuvant, buffering agent, dispersing agent, and the like. A composition will either be in liquid, e.g., in stabilized suspension of the recombinant bacterium, or in solid forms, e.g., a powder of lyophilized recombinant bacteria. E.g. for oral administration, said recombinant bacteria can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups and suspensions. Recombinant bacteria may be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate, and the like.

A preferred composition according to the invention is suitable for consumption by a subject, preferably a human or an animal. Such compositions may be in the form of a food supplement or a food or a food composition, which besides said recombinant bacteria further comprises a suitable food base (i.e., a physiologically acceptable carrier). A food or food composition is herein understood to include a liquid for human or animal consumption, i.e. a drink or beverage. A food or food composition may be a solid, semi-solid and/or liquid food or food composition, and in particular may be a dairy product, such as a fermented dairy product, including but not limited to a yogurt, a yogurt-based drink or buttermilk. Such a food or food composition may be prepared in a manner known *per se*, e.g. by adding said recombinant bacterium to a suitable food or food base, in a suitable amount. In a preferred embodiment, said recombinant bacterium is a microorganism that is used in or for the preparation of a food or food composition, e.g. by fermentation. Examples of such microorganisms are lactic acid bacteria, such as probiotic lactic acid strains as earlier exemplified herein. In doing so, a recombinant bacterium of the invention may be used in a manner known *per se* for the preparation of such fermented food or food compositions, e.g. in a manner known

per se for the preparation of fermented dairy products using lactic acid bacteria. In such methods, the recombinant bacterium according to the invention may be used in addition to a microorganism usually used, and/or may replace one or more or part of a microorganism usually used. For example, in the preparation of a fermented dairy product such as yogurt or yogurt-based drinks, a recombinant lactic acid bacterium of the invention may be added to or used as part of a starter culture or may be suitably added during such a fermentation.

Preferably, the above compositions will contain said recombinant bacterium in amounts that allow for convenient (oral) administration of said recombinant bacterium, e.g. in one or more doses per day or per week. In particular, a composition may comprise a unit dose of said recombinant bacterium.

In another aspect, the present invention is concerned with a recombinant bacterium as defined hereinabove, or a pharmaceutical or nutritional (food) composition comprising such recombinant bacterium, for use as a medicament. Said medicament is preferably for preventing and/or treating an inflammatory GI tract disease, including IBD and ulcerative colitis, in a subject.

The invention also relates to a recombinant bacterium or a composition as defined hereinabove, or a bacterium lacking a polypeptide of a N-acetylgalactosamine phosphotransferase system, lacking a polypeptide encoded by a *lamBDCA* gene cluster, lacking a polypeptide encoded by a bacteriocin gene cluster, lacking a polypeptide encoded by a bacteriocin transport gene cluster, and/or lacking a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, for use as a medicament for treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis.

The invention further relates to a polypeptide of a N-acetylgalactosamine phosphotransferase system, a polypeptide encoded by a *lamBDCA* gene cluster, a polypeptide encoded by a bacteriocin gene cluster, a polypeptide encoded by a bacteriocin transport gene cluster; and/or a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, for use as a medicament.

In an embodiment, a polypeptide of a N-acetylgalactosamine phosphotransferase system may be selected from Enzyme IIA, Enzyme IIB, Enzyme IIC, Enzyme IID and the transcription regulator. In an embodiment, the polypeptide is selected from an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 2, 3, 4, or

5, or homologues thereof having at least 25% identity therewith. Homologues of SEQ ID NOS:2, 3, 4, 5 or 6 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS: 1, 2, 3, 4, or 5, respectively.

- 5 In another embodiment, a polypeptide encoded by a *lamBDCA* gene cluster is selected from the group consisting of histidine protein kinase LamC, response regulator LamA, LamB, and LamD. In an embodiment, the polypeptide is selected from an amino acid sequence selected from the group consisting of SEQ ID NOS: 10, 11, 12, and 13 (LamA, LamC, LamD and LamB, respectively), or a homologue thereof.
- 10 Homologues of SEQ ID NOS: 10, 11, 12, or 13 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS: 10, 11, 12, or 13, respectively.

- A polypeptide encoded by a bacteriocin gene cluster may be selected from the groups consisting of bacteriocins PlnE (SEQ ID NO:8) and PlnF (SEQ ID NO:7), and an immunity protein PlnI (SEQ ID NO:6), , or homologues thereof having at least 25% identity therewith. Homologues of SEQ ID NOS:6, 7, or 8 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:6, 7, or 8, respectively.

- 20 A polypeptide encoded by a bacteriocin transport gene cluster may be any polypeptide selected from bacteriocin ABC-transporter, ATP-binding and permease protein PlnG (SEQ ID NO:9), bacteriocin ABC-transporter, accessory factor PlnH (SEQ ID NO:10), plantaricin biosynthesis protein PlnS (SEQ ID NO:11), and integral membrane proteins PlnT, PlnU, PlnV, and PlnW (SEQ ID NOS:12, 13, 14, and 15, respectively), or homologues thereof having at least 25% identity therewith.
- 25 Homologues of SEQ ID NOS:9, 10, 11, 12, 13, 14, or 15 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NOS:9, 10, 11, 12, 13, 14, or 15, respectively.

- 30 Homologues of SEQ ID NO:20 preferably have at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NO:20.

The skilled person will understand that a polypeptide according to the invention could originate from other hosts than from the herein specified *Lactobacillus plantarum* strain WCFS1, e.g. from other *Lactobacilli* species or even from other probiotic species as long as it has the identity and/or functionality as defined herein.

- 5 Such polypeptide may be obtained using state of the art molecular biology techniques. Most preferably, a polypeptide used is obtained from a *Lactobacillus plantarum* strain. It is also encompassed by the invention to isolate several polypeptides of the invention from one single organism.

- 10 According to another preferred embodiment, a polypeptide of the invention is a variant of any one of the polypeptide sequences defined before. A variant may be a non-naturally occurring form of said polypeptide, which differs in some engineered way from the polypeptide isolated from its native source. Preferably, a polypeptide variant contains mutations that do not alter the biological function of the encoded polypeptide.

- 15 Said polypeptide is intended for use as a medicament, in particular for prevention and/or treatment of inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and/or for stimulation of the immune system. A polypeptide according to the present invention is recovered from cultured host cells and optionally formulated in to a composition suitable for the intended use. Methods for the preparation of such
20 compositions are known per se, and are further illustrated hereinabove in respect of compositions comprising said recombinant bacterium.

- In another aspect, the invention relates to a method for (site-specific) production of a polypeptide of the invention at a mucosal surface of a subject as has been exemplified in WO 05/040387. The method comprises the step of administering to the
25 subject a composition comprising a polypeptide as defined above and/or a recombinant bacterium of the invention.

- Also, the invention pertains to a nucleic acid construct comprising one or more genes encoding: a) a polypeptide of a N-acetylgalactosamine phosphotransferase system; b) a polypeptide encoded by a *lamBDCA* gene cluster, c) a polypeptide
30 encoded by a bacteriocin gene cluster, d) a polypeptide encoded by a bacteriocin transport gene cluster; and/or e) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, operably linked to a promoter, for use as a medicament, in particular for prevention and/or treatment of

inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and/or for stimulation of the immune system. Such polypeptides may be any polypeptide as defined hereinabove, including homologues and variants thereof having at least 25% identity therewith. Nucleic acid sequences encoding the polypeptides of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 may be found in publicly available databases, as will be known by the skilled person. However, said nucleic acid construct may comprise a variant of these nucleic acid sequences. Such nucleic acid variant may e.g. be a nucleic acid sequence that differs from the nucleic acid sequences set forth in the sequence listing by virtue of the degeneracy of the genetic code. E.g., the genetic code of such nucleic acid sequence may be optimized for expression in a particular host organism. Nucleic acid sequence variants may be obtained using techniques known to the skilled person.

A nucleic acid construct of the invention comprises a nucleic acid sequence encoding a polypeptide operably linked to a promoter, and optionally one or more further control sequences, which direct the production of a polypeptide in a suitable expression host. "Expression" will be understood to include any step involved in the production of a polypeptide including, but not limited to transcription, post-transcriptional modification, translation, post-translational modification and secretion. A "nucleic acid construct" is defined as a nucleic acid molecule, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined or juxtaposed in a manner which would not otherwise exist in nature. A "control sequence" is defined herein to include all components which are necessary or advantageous for the expression of a polypeptide. At a minimum, the control sequence include transcription and translational stop signals in addition to a promoter.

Said nucleic acid construct is intended for use as a medicament, in particular for prevention and/or treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis, and/or for stimulation of the immune system. Such nucleic acid construct may be formulated into composition suitable for such intended use. Methods for the preparation of such compositions are known per se, and are further illustrated hereinabove in respect of compositions comprising said recombinant bacterium and/or polypeptide of the invention. Said nucleic acid construct may be a chimeric gene encompassing one or more genes of the invention in combination with one or more

promoters that these are not naturally associated with. The promoter may be a constitutive or an inducible promoter.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, the verb "to consist" may be replaced by "to consist essentially of" meaning that a composition of the invention may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristics of the invention.

The word "approximately" or "about" when used in association with a numerical value (approximately 10, about 10) preferably means that the value may be the given value of 10 plus or minus 1% of the value.

In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". It is further understood that, when referring to "sequences" herein, generally the actual physical molecules with a certain sequence of subunits (e.g. amino acids) are referred to.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

It will also be clear that the description and examples are included merely to illustrate some embodiments of the invention, and not to limit the scope of protection. Starting from this disclosure, many more embodiments will be evident to a skilled person, which are within the scope of protection and the essence of this invention and which are obvious combinations of prior art techniques and the disclosure of this patent.

Sequence Listing

Table 1. Sequences set forth in the Sequence Listing appended. SEQ ID Nos: 1-13 represent amino acid sequences, whereas SEQ ID Nos: 14-26 represent nucleic acid sequences.

SEQ ID NO.	Locus tag	Entry	Description
1	Lp_2647	CAD64902	Pts19A N-acetylglucosamine/galactosamine PTS, EIIA
2	Lp_2648	CAD64903	Pts19D N-acetylgalactosamine-specific PTS system transporter

			subunit IID
3	Lp_2649	CAD64904	Pts19CN-acetylgalactosamine PTS, EIIC
4	Lp_2650	CAD64905	Pts19B N-acetylgalactosamine-specific PTS system transporter subunit IIB
5	Lp_2651	CAD64906	Transcription regulator
6	Lp_0419	CAD63054	PlnI immunity protein PlnI
7	Lp_0421	CAD63055	PlnF bacteriocin precursor peptide PlnF (putative)
8	Lp_0422	CAD63056	PlnE bacteriocin precursor peptide PlnE (putative)
9	Lp_0423	CAD63057	PlnG bacteriocin ABC-transporter, ATP-binding and permease protein PlnG
10	Lp_0424	CAD63058	PlnH bacteriocin ABC transporter, accessory factor
11	Lp_0424a	CAD63059	PlnS plantaricin biosynthesis protein
12	Lp_0425	CAD63060	PlnT integral membrane protein
13	Lp_0426	CAD63061	PlnU integral membrane protein
14	Lp_0428	CAD63062	PlnV integral membrane protein
15	Lp_0429	CAD63063	PlnW integral membrane protein
16	Lp_3580	CAD65658	agrA response regulator; accessory gene regulator protein A
17	Lp_3581	CAD65659	agrC histidine protein kinase; sensor protein
18	Lp_3581a	CAD65660	agrD accessory gene regulator protein D
19	Lp_3582	CAD65661	agrB accessory gene regulator protein B
20	Lp_2991	CAD65174	Transcription regulator

Examples

Example 1 Immunomodulation of Peripheral blood mononuclear cells (PBMCs)

5

Materials and methods

Bacterial strains

A total of 42 *L. plantarum* strains isolated from different gut and food sources were examined used for immunoprofiling and comparative genome hybridization (Table 1). Genomic comparisons of twenty of these strains was performed previously (Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem. 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J Bacteriol 187:6119-6127). Comparative genome hybridization of an additional 22 strains with distinct phenotypic profiles was performed using methods

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described by Molenaar *et al.* (*supra*)(Tzeneva *et al.*, personal communication). For immunoprofiling, *L. plantarum* was grown at 37°C in MRS until exponential phase (optical density (OD) 600nm = 1) or overnight for stationary phase cells. The cultures were washed twice in phosphate buffered saline (PBS, pH 7.4), resuspended at 2×10^8 cells/ml in PBS containing 20% glycerol, and stored at -80°C until immunoprofiling. Colony forming units (CFUs) were determined by plating serial dilutions of the cultures on MRS agar. Unless indicated otherwise, stationary grown bacteria were used for immunoprofiling.

10 Peripheral blood mononuclear cells assay

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the peripheral blood of healthy donors using a Ficoll-paque Plus (Amersham biosciences, Uppsala, Sweden) gradient centrifugation according to the manufacturer's protocol. After gradient centrifugation the mononuclear cells were collected, washed in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax (Invitrogen, Breda, The Netherlands) and adjusted to 1×10^6 cells/ml in IMDM + glutamax supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml) (both Invitrogen) and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1×10^6 cells/ml) were seeded in 48-well tissue culture plates. After an overnight rest at 37°C in 5% CO₂, 5 µl aliquots of thawed bacterial suspensions at 2×10^8 CFU/ml were added to the PBMCs (*L. plantarum* :PBMC ratio of 1:1). After 24 hr incubation at 37°C in 5% CO₂, culture supernatants were collected and stored at -20°C for cytokine analysis. Neither medium acidification nor bacterial proliferation was observed (data not shown). Cytokines were measured by BD Cytometric Bead Array Flexsets (BD Biosciences, Franklin Lakes, New Jersey) for interleukin(IL)-10 , IL-12, IL6, IL1β TNFα and interferon- γ, according to the manufacturer's recommendations. Concentrations of analytes were calculated with the use of known standards and plotting of the samples against a standard curve using the FCAP 2.0 software. In total, three blood samples from different donors and two independently grown cultures of each *L. plantarum* strain were examined for modulation of cytokine secretion by PBMCs. To compare amounts of cytokines produced by the different donors, the levels for *L. plantarum* WCFS1 were set at 100% and within each donor the cytokine levels induced by the other strains were related to strain WCFS1.

Identification of candidate genes involved in cytokine secretion by gene-trait matching

Candidate *L. plantarum* genes with potential roles in induction of cytokine secretion by PBMCs were identified by *in silico* gene-trait matching (Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P. A. Bron, J. Lambert, W. M. de Vos, R. van der Meer, M. A. Smits, and M. Kleerebezem. 2005. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *Journal of Bacteriology* 187:6128-6136) using genotype information referenced from the *L. plantarum* WCFS1 genome. Correlations between gene presence/absence patterns in the *L. plantarum* strains (Molenaar *et al.*, *supra*) and IL-10 or IL-12 concentrations or concentration ratios excreted by the PBMCs were investigated by regression using the Random Forest algorithm (Breiman, L. 2001. Random forests. *Machine Learning* 45:5-32). An implementation of the method in the “RandomForest” package for R (Liaw, A., and M. Wiener. 2002. Classification and regression by randomForest. *R news*, <http://www.r-project.org> 2:18-22) was used with standard parameter settings. In this approach, gene presence-absence patterns for the 42 strains was used as a putative predictor variable for the interleukin concentration or concentration ratio. *L. plantarum* WCFS1 genes with the highest variable importance measures as returned by the Random Forests method were selected for deletion analysis to determine their roles in induction of interleukin production by PBMCs.

Construction of knock-out mutants

A previously described *L. plantarum* $\Delta lamAR$ mutant (Fujii, T., C. Ingham, J. Nakayama, M. Beerthuyzen, R. Kunuki, D. Molenaar, M. Sturme, E. Vaughan, M. Kleerebezem, and W. de Vos. 2008. Two Homologous agr-like Quorum Sensing Systems co-operatively Control Adherence, Cell morphology, and Cell Viability Properties in *Lactobacillus plantarum* WCFS1. *J Bacteriol*) was used in this study. Construction of the *L. plantarum* gene deletion mutants for the following genes: lp_1953, lp_2647-2651, lp_0419-0422 and lp_0423 was performed as previously described with several modifications (Lambert, J. M., R. S. Bongers, and M. Kleerebezem. 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl Environ Microbiol* 73:1126-1135). Flanking primers were used to amplify the 5' and 3' ends of the selected genes (Table

2) and the regions flanking the gene of interest (approximately 1 kb on each side). The lox-cat-lox region of pNZ5319 was amplified using primers Ecl-loxR and Pml-loxF. In a so-called SOEing reaction (Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* 8:528-535), the three PCR products were linked to each other due to overlapping regions in the primers. PCR products were cloned into the non-replicating integration vector pNZ5319 (Lambert, J. M., R. S. Bongers, and M. Kleerebezem. 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl Environ Microbiol* 73:1126-1135) after digestion of the vector with *Swa*I and *Ecl*136II. Plasmids were transformed into competent cells of *E. coli* JM109 by electroporation as recommended by the manufacturer (Invitrogen). This resulted in a plasmid containing the complete gene replacement cassette. Plasmid DNA was isolated from *E. coli* by using Jetstar columns, following the manufacturer's instructions (Genomed GmbH, Bad Oeynhausen, Germany). The sequence of the genes cloned were confirmed by sequence analysis (BaseClear, Leiden, The Netherlands).

L. plantarum WCFS1 was transformed by electroporation and integrants were selected by plating the resulting bacteria on MRS agar supplemented with chloramphenicol ($10 \mu\text{g ml}^{-1}$) and incubation at 37°C for 2 to 4 days. Plasmid excision was confirmed by measuring erythromycin sensitivity ($30 \mu\text{g ml}^{-1}$) of individual isolates and correct integration was confirmed by colony PCR using primers flanking the sites of recombination. To excise the P_{32} -cat selectable-marker cassette from the chromosome, the replacement mutants were transformed with the transient erythromycin-selectable *cre* expression plasmid pNZ5348. After a PCR check for Cre-mediated recombination, the pNZ5348 vector was cured from appropriate colonies of *L. plantarum* mutants.

Results

Immunomodulation of PBMCs is a variable phenotype in *L. plantarum*

Cytokine amounts induced by the 42 *L. plantarum* strains were donor-dependent, especially for IL-12. For PBMCs isolated from donor A the measured values for IL-12 ranged from 52 to 600 pg/ml, while for donor B the measured values

were between 2 to 60 pg/ml. However, the bacterial strains with strong cytokine stimulating properties in one donor had also induced high levels of cytokines in the other donor ($R^2 = 0.6$). Moreover, variation between the bacterial strains in their capacity to stimulate PBMCs was consistent and not dependent of the donor. For IL-10 the variation between the strains was 8-fold, whereas a 16-fold difference between strains was found for IL-12 and 9-fold for the IL10/IL12 ratios. Compared with the other *plantarum* strains *L. plantarum* WCFS1 conferred a relatively low IL-10 stimulating capacity, high IL-12 stimulating capacity, and low IL-10/IL-12 ratio.

The measurements of IL-10 and IL-12 levels as well as the ratio between these cytokines are widely used to describe anti- and proinflammatory properties of bacteria in PBMC assays. *L. plantarum* WCFS1 induced the secretion of other cytokines including $TNF\alpha$, $IFN\gamma$, IL6 and IL1 β by PBMCs (data not shown), however because levels of these cytokines correlated well with IL-12 amounts ($R^2 = 0.8-0.9$) these proteins were not included in subsequent analyses.

Identification of candidate genes involved in immunomodulation

PBMCs stimulating capacities of the *L. plantarum* strains were compared to the comparative genome hybridization (CGH) profiles of the same strains to identify candidate *L. plantarum* WCFS1 genes involved in modulation of PBMC responses.

Correlation of the IL-10 levels elicited by the *L. plantarum* strains to gene presence/absences scores against the *L. plantarum* WCFS1 genome resulted in the identification of 6 genes with putative roles in IL-10 stimulation. These 6 genes were divided in two clusters: strains containing homologs of lp_1953 showed, on average, a 1.6-fold higher IL-10 stimulating capacity compared to strains without these genes.

Lp_1953 is encoding a hypothetical protein with an unknown function, predicted to be located intracellular (Zhou, M., J. Boekhorst, C. Francke, and R. J. Siezen. 2008. LocateP: genome-scale subcellular-location predictor for bacterial proteins. BMC Bioinformatics 9:173) and the homolog is present in 48% of the tested strains. Strains containing homologs of the multi-gene locus lp_2647 to lp_2651 induced, on average, a 1.7x lower IL-10 stimulating capacity compared to strains without these genes. Lp_2647 to lp_2651 encode Pts19ADCBR, a *N*-acetyl-galactosamine phosphotransferase system and putative transcription regulator. Homologs of this operon are present in 33% of the tested strains. Correlations between CGH data and IL-

12 levels among the *L. plantarum* strains did not result in identification of specific genes which might modulate expression levels of this cytokine in PBMCs. The Random Forests method returned no genes with high variable important measures, indicating that other factors than presence/absence of the genes in the WCFS1 genome are responsible for the observed variation between bacterial strains.

Comparisons between the CGH data and IL-10/IL-12 ratios induced by the *L. plantarum* strains, resulted in the identification of 7 genes for which the presence/absence profile in the bacterial strains correlated with the IL-10/IL-12 ratio. Lp_0419 to lp_0423 and lp_3582 were predominantly present in strains stimulating a low IL10/IL12 ratio. Lp_0419 to lp_0422 are the *plnEFI* operon, encoding two bacteriocin-like peptides and a bacteriocin immunity protein and homologs of the genes in this operon are present in 81-85% of the tested strains. Lp_0423 is distal to lp_0422, located in another operon, and encodes an ABC transporter involved in the transport of bacteriocins (Diep, D. B., L. S. Havarstein, and I. F. Nes. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol 178:4472-4483). Lp_0423 is present in 88% of the tested strains. Lp_3582 encodes an accessory gene regulator protein B (LamB) of the *lamBDCA* operon. This operon encodes for the *Lactobacillus* agr-like quorum sensing module important for biofilm formation and regulation of adherence (Sturme, M. H., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, T. Fujii, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos. 2005. An agr-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J Bacteriol 187:5224-5235). A homolog of lp_3582 is present in 33% of the tested strains.

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Verification of the roles of the candidate genes in immunomodulation

To validate the contributions of the *L. plantarum* candidate genes in modulating PBMC responses, mutants of *L. plantarum* WCFS1 with gene-specific deletions were constructed for lp_1953, *pts19ADCBR*, *plnEFI* and *plnG*. *L. plantarum* WCFS1 Δ *lamAR* (lp_3580 and lp_3087) was used to examine the potential roles of *lamB* on PBMCs. This mutant expresses significantly lower amounts of all genes in the *lamBDCA* operon (Fujii, T., C. Ingham, J. Nakayama, M. Beerthuyzen, R. Kunuki, D. Molenaar, M. Sturme, E. Vaughan, M. Kleerbezem, and W. de Vos. 2008. Two

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Homologous agr-like Quorum Sensing Systems co-operatively Control Adherence, Cell morphology, and Cell Viability Properties in *Lactobacillus plantarum* WCFS1. J Bacteriol). The deletion mutants were compared to the wild-type strain for the capacity to stimulate IL-10 and IL-12 in PBMCs. Because *L. plantarum* WCFS1 was previously
 5 shown to differentially modulate human duodenal cell responses *in vivo* in ways which were dependent on the growth-phase of the *L. plantarum* cells (van Baarlen, P., F. J. Troost, S. van Hemert, C. van der Meer, W. M. de Vos, P. J. de Groot, G. J. Hooiveld, R. J. Brummer, and M. Kleerebezem. 2009. Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating
 10 with immune tolerance. Proc Natl Acad Sci U S A 106:2371-2376), both exponential and stationary-phase cultures of the wild-type and mutant strains were examined for their immunomodulatory effects.

Stationary phase cultures of *L. plantarum* Δ *pst19ADCBR* stimulated PBMCs to secrete higher IL-10 amounts (25 – 50%, depending on the donor, $p < 0.01$) than wild-
 15 type *L. plantarum* WCFS1. This result is in agreement with the CGH gene-trait matching comparisons which predicted that *L. plantarum* strains lacking Pst19ADCBR confer higher PBMC IL-10 secretion levels. *L. plantarum* Δ *pst19ADCBR* induced the same amounts of IL-12 by the PBMCs as the wild-type *L. plantarum* WCFS1 strain. When logarithmic phase cultures were tested, no difference in stimulation of PBMCs
 20 was observed between wild-type *L. plantarum* WCFS1 and *L. plantarum* Δ *pst19ADCBR*.

In contrast, deletion of the *lp_1953* gene from *L. plantarum* WCFS1 resulted in no significant change in IL-10 or IL-12 amounts. Although somewhat higher amounts of both cytokines were found for the mutant compared to wild-type cells, these
 25 differences were not significant.

Among the 42 *L. plantarum* strains tested, the strains containing homologs of *L. plantarum* WCFS1 genes *plnEFI*, *plnG* or *lamB* predominantly conferred lower PBMC IL-10/IL-12 ratios compared to strains lacking those genes (Table 3). To confirm the roles of these genes in directing PBMC responses, *L. plantarum* WCFS1 *plnEFI* and
 30 *plnG* deletion mutants were examined for stimulation of IL-10 and IL-12 production by PBMCs. Both mutants induced higher IL10/IL12 ratios compared to PBMCs stimulated with the wildtype *L. plantarum* WCFS1. The PBMCs responses were significantly different for only *L. plantarum* cells harvested during active, exponential phase growth

and not stationary phase. The ratios were largely affected by the increased IL-10 response by PBMCs to the exponential-phase *L. plantarum* mutants, although the IL-12 normalized comparisons were important to clearly distinguish the mutants from wild-type *L. plantarum* WCFS1 cells.

- 5 A similar result was found for exponential-phase cells of *L. plantarum* $\Delta lamAR$. This strain induced higher IL-10/IL-12 ratios compared to *L. plantarum* WCFS1, a result which was significantly affected by the higher IL-10 levels induced by the *L. plantarum* $\Delta lamAR$ strain. Surprisingly, stationary phase *L. plantarum* $\Delta lamAR$ induced significantly higher amounts of IL-10 and IL-12 from the PBMCs, but because these
10 cytokines were similarly affected the IL-10/IL-12 ratio remained unaffected.

Example 2. Immunomodulation of dendritic cells (DCs)

Material and methods

15 *Bacterial strains*

- 42 different *L. plantarum* strains isolated from humans and different food resources were used for immunoassays and comparative genome hybridization studies (CGH) (Table 1). Lactobacilli were grown overnight to stationary phase at 37°C in MRS. The bacteria were recovered by centrifugation and washed twice in phosphate buffered
20 saline (PBS, pH=7.4) and resuspended at 2×10^8 CFU/ml in PBS containing 20% glycerol and stored at -80°C prior to use in the immunoassays. Colony forming units (CFU) were determined by plating serial dilutions of the cultures on MRS agar.

Blood donors

- 25 Buffy coats from blood donors were obtained from the Sanquin Blood bank Nijmegen (The Netherlands). An informed consent was obtained before the sample collection and the performed experiments were approved by the Local Ethical Committee.

Differentiation and maturation of dendritic cells

- 30 Human peripheral blood mononuclear cells (PBMCs) were isolated from blood using a combination of Ficoll density centrifugation and cell separation using antibody coated magnetic microbeads. The blood was diluted 1:1 with Iscove's Modified Dulbecco's Medium (IMDM) containing GlutaMAX (Invitrogen). The PBMCs were isolated by

density gradient centrifugation on Ficoll-Plaque PLUS (GE Healthcare). The diluted plasma was removed and the layer of white blood cells were carefully recovered using a pipette and then washed twice with IMDM. The CD14⁺ monocytes were then purified using magnetic cell sorting CD14⁺ microbeads according to the manufacturers recommended protocols (Miltenyi Biotec). The proportion of CD14⁺ cells was routinely determined using flow cytometry (BD FACSCanto II). In all experiments the proportion of CD14⁺ cells was greater than 80%. To generate immature DC approximately 10⁶ CD14⁺ cells / well) were cultivated in RPMI 1640 containing 10% FBS gold (PAA), 1% penicillin, streptomycin (v/v) (Invitrogen), IL-4 (50 ng/ mL, R&D systems) and GM-CSF (50 ng/ mL, R&D systems) in a 24 well plates. GM-CSF combined with IL-4 drives monocytes to become myeloid dendritic cells *in vitro* in 6 days. At day 3 and day 6 half of the medium was refreshed. At day 6 the cells were left unstimulated (immature DCs (iDCs)) or were stimulated with LPS (1 µg/ mL) or with different *L. plantarum* strains or WCFS1 deletion mutants (1:1 bacteria to DC ratio) for 48 hours. Over this period of time no acidification of the medium or bacterial proliferation was observed.

Analysis of cell surface markers and measurement of cell death by flow cytometry

Monocyte-derived dendritic cells were harvested at day 3, day 6 and day 8 and were stained with specific monoclonal antibodies to CD83, CD86 or their isotype-matched controls (BD biosciences, San Diego, USA) for 30 min on ice, washed and analyzed by flow cytometry (FACSCanto II, BD, San Diego, USA). To check the activation status of the cells (data of day 3 and 6 not shown), the CD86 expression on the cells are measured. CD83 is only expressed on matured dendritic cells, i.e. fully activated dendritic cells.

Live, apoptotic and necrotic cells were discriminated by staining with annexin V and propidium iodide at day 3, day 6 and day 8. Cells were washed and subsequently incubated with 2 µl Annexin V-APC (BD biosciences, San Diego, USA) in 200 µl Annexin V buffer according to the manufacturer's protocol. After an incubation period of 15 min on ice, the cells were spun down (300g for 10 min) and resuspended in 200 µl Annexin V buffer plus 2 µl propidium iodide (1 mg/ml; Sigma). The cells were thereafter analyzed on a flow cytometer (FACSCanto II, BD, San Diego, USA). Cells that are negative for both Annexin V and PI are not apoptotic or necrotic as

translocation of the membrane phospholipid phosphatidylserine has not occurred and the plasma membrane is still intact. Therefore, Annexin V and PI double negative cells were considered as viable cells, whereas both single and double positive cells were regarded as non-viable (Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods **184**:39-51). The flow cytometry data was analysed using the BD FACSDiva software. The viability of the cells was between 50- 80%.

10 *Cytokine analysis*

Supernatants from the DC stimulation assays were collected after 48 hours incubation, stored at -20 °C and cytokines measured within 2 weeks using a cytometric bead-based immunoassay that enables multiplex measurements of soluble cytokines in the same sample (Morgan et al. 2004. Clin. Immunol. 110:252-266). The cytokines TNF- α , IL-12p70 and IL-10 were measured after 48h incubation using cytometric bead array flex sets, the FACS Canto II cytometer and the buffers and protocols recommended by the manufacturer (BD biosciences). According to the manufacturer the limits of sensitivity for detection were as follows: TNF- α 0.7 pg/mL; IL-12p70 0.6 pg/mL and IL-10 0.13 pg/mL. The flow cytometry data was analysed using the BD FCAP software.

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Identification of candidate genes involved in cytokine secretion by gene-trait matching

Candidate *L. plantarum* genes, that were potentially involved in modulation of the DC responses were identified by *in silico* gene-trait matching (Pretzer et al. 2005. J. Bacteriol. 187:6128-6136) using genotype information referenced from the *L. plantarum* WCFS1 genome. The significance of the gene-trait co-occurrence was assessed by assuming a discrete probability distribution of genes and traits in the context of a null hypothesis that co-occurrence is caused by a random process (Jim et al. 2004. Genome Res. 14:109-115). All *L. plantarum* genes were tested for their significant co-occurrence with each cytokine concentration or cytokine concentration ratio (i.e. IL-10/IL-12). *L. plantarum* WCFS1 genes with the highest variable importance measures as returned by the Random Forests method were selected for further characterization using genetic approaches in combination with immunoassays.

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Construction of knock-out mutants

A previously described *L. plantarum* lp_3536 deletion mutant (Lambert et al. 2007. Appl. Environ. Microbiol. 73:1126-1135) was used in this study. Construction of the *L. plantarum* gene deletion mutants for the following genes: lp_0419-0422, lp_0423, 5 lp_0423-30 and lp_2991 was performed as previously described with several modifications (Lambert et al. 2007, *supra*). Flanking primers were used to amplify the 5' and 3' ends of the selected genes (Table 2) and the regions flanking the gene of interest (approximately 1 kb on each side). The lox-cat-lox region of pNZ5319 was amplified using primers Ecl-loxR and Pml-loxF. In a so-called SOEing reaction 10 (Horton et al. 1990. Biotechniques 8:528-535), the three PCR products were linked to each other due to overlapping regions in the primers. PCR products were cloned into the non-replicating integration vector pNZ5319 (Lambert et al. 2007, *supra*) after digestion of the vector with SmaI and Ecl136II. Plasmids were transformed into competent cells of *E. coli* JM109 by electroporation as described by the manufacturer 15 (Invitrogen). This resulted in a plasmid containing the complete gene replacement cassette. Plasmid DNA was isolated from *E. coli* by using Jetstar columns, following the manufacturer's instructions (Genomed GmbH, Bad Oeynhausen, Germany). The sequence of the cloned DNA was confirmed by sequence analysis (BaseClear, Leiden, The Netherlands).

20 *L. plantarum* WCFS1 was transformed by electroporation as previously described and integrants were selected by plating the resulting bacteria on MRS agar supplemented with chloramphenicol (10 $\mu\text{g ml}^{-1}$) and incubation at 37°C for 2 to 4 days. Plasmid excision was confirmed by measuring erythromycin sensitivity (30 $\mu\text{g ml}^{-1}$) of individual isolates and correct integration was confirmed by colony PCR using primers 25 flanking the sites of recombination .

Statistical analysis

Mixed general linear model using restricted maximum likelihood (REML) was used to determine the statistical differences within donors between cytokine secretion by DCs 30 stimulated with the constructed deletion mutants compared to the wild type *L. plantarum* WCFS1. A two-sided *p*-value of 0.05 or lower was considered to be significant. The statistical analysis was performed by using SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA)

Results

DC cytokine responses to different L. plantarum strains

5 Monocyte derived immature dendritic cells from five different healthy donors were cultured in the presence of 20 different *L. plantarum* strains. The strains differed considerably in their ability to modulate DC responses. For example, the amounts of IL-10 induced by the strains varied from 28 pg/ml to 1095 pg/ml (39 fold) and for IL-12 the values ranged from 20-11996 pg/ml (600 fold). For TNF-alpha some strains
10 induced very low amounts (close to the detection limit – 0.7 pg/mL) whereas others induced 8.4 to 12 ng/ml. The large variation in strain immune profiles suggest that there is some underlying genetic variation influencing the innate response to *L. plantarum*.

Some strains such as B2766, B2801 and B2897 were clearly strong inducers of pro-inflammatory cytokines IL-12 and TNF-alpha while others were considerably less
15 potent (e.g. strains B1839, B2494 and B2831). Similarly, the strains showed strikingly different capacities to induce the anti-inflammatory cytokine IL-10. From a comparison of IL-12 to IL-10 ratios it is clear that these cytokines can vary independently of each other allowing the possibility for strains with distinct pro-inflammatory (e.g. strain
20 B1840 and B2257) and anti-inflammatory profiles (e.g. strain CIP104448). As expected levels of cytokines induced by *L. plantarum* strains differed between donors but the ranking of the strains was highly consistent for each cytokine showing that the strain immunoprofiles were reproducible. Immune responses to strain B1839 were strikingly lower than for all other strains suggesting that it might directly attenuate immune
25 responses, possess non-typical MAMPs or EPS that prevent innate recognition. The strain differences observed in these experiments were not due to variation in CFU of bacteria as the samples used in the assays were checked twice by plating on solid medium and measurements of optical density (OD_{600nm}).

30 *Identification of candidate gene loci by in silico gene trait matching*

L. plantarum genes potentially involved in the production of pro and anti-inflammatory cytokines were identified by *in silico* gene-trait matching by correlating measurements of cytokines induced by the different strains with genotypic information available for

the same strains. Seven genes displayed a match with lower levels of IL-10 concentration in the co-culture system. One of these genes, *lp_2991* is annotated as a transcription regulator which is present in 90% of the strains tested. The other six genes (*lp_0422*, *lp_0423*, *lp_0424a*, *lp_0424*, *lp_0425* and *lp_0429*) lie within the multigene locus (*lp_0422* to *lp_0429*) involved in plantaricin biosynthesis and secretion. The *plnEFI* operon (*lp_0419* to *lp_0422*) is encoding two bacteriocin-like peptides and a bacteriocin immunity protein. Homologues of the gene loci in this operon are present in 81-85% of the tested strains. *Lp_0423* is distal to *lp_0422* and located in another operon and encodes an ABC transporter involved in the transport of bacteriocins (10, 44). *Lp_0423* (*plnG*) is present in 88% of the tested strains.

Three genes (*lp_2991*, *lp_0422* and *lp_3536*) had gene-trait match with a lower concentration of TNF- α produced in the supernatant of *L. plantarum* DC co-culture. There was a co-occurrence of low TNF- α secretion and the presence of *lp_2991* and *lp_3536*. *Lp_3536* is predicted to encode a bile salt hydrolase capable of removing the amino acid moiety from the steroid nucleus of conjugate bile salts by hydrolysis and is present in 81% of the tested strains.

Validation of the role of the candidate genes in cytokine secretion

To validate the role of the genes identified by gene-trait matching in modulating DC cytokine secretion, specific deletion mutants of genes *lp_0423-0429*, *lp_2991*, *lp_0419-0422*, *lp_0423* and *lp_3536* were constructed in *L. plantarum* strain WCFS1, to yield strains *lp_2991::cat*, *plnEFI::cat*, *plnG::cat*, *plnGHSTUVWXWX::cat*. Construction of the *lp_3536::lox72* mutant was described previously. The capacity of the deletion mutants to induce IL-10, IL-12p70 and TNF- α was then compared to the wild-type strain *L. plantarum* WCFS1. As expected deletion mutants lacking genes involved in plantaricin secretion and immunity induced significantly higher amounts of IL-10 in DC co-culture compared to the wild type strain WCFS1 (Table 4). For mutant *plnEFI::cat* in which the two bacteriocin-like peptides and a bacteriocin immunity protein were deleted, IL-10 was significantly increased 3.3. fold ($p < 0.05$). Deletion of the pheromone and bacteriocins transport operon (*plnGHSTUVWX*), in strain *plnGHSTUVWX::cat* also significantly increased IL-10 3.1-fold ($p < 0.05$) compared to the wild type strain WCFS1. Similar increases (3.2 –fold; $p < 0.05$) were also observed for *lp_0423::cat* lacking *plnG*.. In the *plnEFI*, *plnGHSTUVWX* and *plnG* mutants TNF-

α secretion was significantly increased by 4.2-fold ($p<0.05$) and 7.4 fold ($p<0.05$) respectively. In all plantaricin associated mutants IL-12p70 secretion was also significantly ($p<0.05$) increased between 1.9 - 2.4 fold.

The presence of the *lp_2991* gene in strains was associated with induction of lower amounts of IL-10 and TNF-alpha secretion compared to strains lacking this gene. As expected deletion of this gene in wild type strain WCFS1 significantly increased IL-10 and TNF-alpha secretion compared to the wild type strain. IL-10 secretion was increased 6.3-fold ($p<0.05$) and TNF-alpha secretion was increased 17.2-fold ($p<0.05$). Additionally, IL-12p70 secretion was induced 3.2-fold ($p<0.05$). Deletion of *lp_3536* (strain *lp_3536::loxp72*) had no significant effect on cytokine production compared to the wt strain.

TLR2/6 signalling assay

To investigate the role of TLR2/6 activation in the DC cytokine responses to different *L. plantarum* strains, a human embryonic kidney cell line (HEK293) was constructed that stably expressed human TLR2/6 carrying a reporter plasmid (pNiFTY) containing firefly luciferase under the control of the human NF-kB promoter. HEK293 cells do not produce TLRs, but when stably transformed with a TLR2/6 expression plasmid, they can activate NF-kB upon addition of Pam(3)Cys-SK4 (PCSK), a known synthetic agonist of this receptor. TLR2/6 transfected cells were seeded at 5×10^5 cells/cm². Cells were challenged with either Pam3CSK4 5µg/ml or WCFS1 or the *lp_2991* deletion mutant (1:1.5, 1:5, 1:15, 1:50 1:150 and 1:450 cell to bacteria ratio). Two independent assays were run with six technical replicates. After six hours incubation the medium was replaced with Bright-Glo luciferase assay buffer (Promega) and luminescence intensity was measured in a Spectramax M5 reader (Molecular devices) within 15 minutes. The *lp_2991* deletion mutant was less capable in activating TLR2/6 (about 2-3 fold lower NF-kB activation compared to WCFS1).

TABLES

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Table 1. Origin of bacterial strains used in the studies

Strain #	Received as	Isolation source	Geographical origin
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NIZO1836	WCFS1	Human saliva	England
NIZO2263	LP80	Silage	n.a.
NIZO2814	Lp95	Wine red grapes	Italy
CIP102359	CIP102359	Human spinal fluid	France
NIZO2726	ATCC8014	Maize ensilage	n.a.
NIZO2891	LD3	Radish pickled	Vietnam
		Pork pickled sour	
NIZO2457	CHEO3	sausage	Vietnam
NIZO2535	LD2	Orange fermented	Vietnam
NIZO2830	BLL(EI31)	n.a.	n.a.
NIZO2259	CIP104452	Human tooth abscess	France
NIZO2831	CECT221(24Ab04)	Grass silage	United States
NIZO2262	LM3	Silage	n.a.
		Pork pickled sour	
NIZO2494	NCTH27	sausage	Vietnam
NCDO1193	NCDO1193	Vegetables	n.a.
NIZO2806	LMG9208	Sauerkraut	United Kingdom
NIZO2896	ATCC14917^a	Cabbage pickled	Denmark
NIZO2741	NOS140	Cabbage kimchi	Japan
NIZO1837	299	Human colon	United Kingdom
		Pork pickled sour	
NIZO2855	N58	sausage	Vietnam
NIZO2877	X17	Hot dog	Vietnam
NIZO2260	299v/DSM9843	Human intestine	United Kingdom
NIZO2029	MLC43	Raw cheese with rennet	Italy
NIZO2889	LAC7	Banana fermented	Vietnam
NIZO2264	LP85-2^b	Silage	France
		Pork pickled sour	
NIZO2484	NCTH19-1	sausage	Vietnam
		Pork pickled sour	
NIZO2485	NCTH19-2	sausage	Vietnam
NIZO2261	NC8	Grass silage	Sweden
NIZO2802	KOG24	Cheese	Japan
NIZO2801	KOG18	Turnip pickled	Japan
NIZO3400	LMG18021	Milk	Senegal
NIZO2753	Q2	Sourdough fermented	Italy

NIZO1839	SF2A35B^b	Sour cassava	South America
NIZO2258	CIP104451	Human urine	France
NIZO2257	CIP104450	Human stool	France
CIP104448	CIP104448	Human stool	France
NIZO2897	DKO22 ^b	Sour cassava	Nigeria
NIZO2766	H14	Sourdough fermented	Italy
NIZO2757	H4	Sourdough fermented	Italy
NIZO2776	CECT4645	Cheese	n.a..
NIZO2256	CIP104441	Human stool	France
NIZO1838	CIP104440	Human stool	France
NIZO1840	NCIMB12120^b	Cereal fermented (Ogi)	Nigeria

n.a. not available

Strains in bold were also compared in Molenaar et al (2005). The other strains were new in this study.

^a Draft genome sequence available April 2009 (NZ_ACGZ000000000.1).

^b Putative subspecies *argenteratensis*

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TABLE 2. Primers used for preparing mutants

Primer	sequence
LF1953F	5'- TGCCGCATACCGAGTGAGTAG -3'
LF1953R	5'- <u>CGAACGGTAGATTTAAATTGTTT</u> ATCAAAAACACCGTTAATTTGCATC -3'
RF1953F	5'- <u>GTACAGCCCGGGCATGAG</u> CGTGGCCATTAGTTGACGAGAC -3'
RF1953R	5'- AACGCCATCGCACTGATGCATC -3'
Ecl-loxR	5'- AAACAATTTAAATCTACCGTTTCG -3'
Pml-loxF	5'- CTCATGCCCCGGGCTGTAC -3'
LF1953F2	5'-GCAACGGCTGTCAGTAACCTGCCTTC-3'
RF1953R2	5'-TCAAATCTCGAAGCGGTTCAAACACTG-3'
LF2647F	5'- <u>GTACAGCCCGGGCATGAG</u> GGTATTTAGCGAAATATACAGATTG -3'
LF2647R	5'- CTTTAGCCGTCTCATTAGTCG -3'
RF2651F	5'- GGATTACCAAAACGAACATGG -3'
RF2651R	5'- <u>CGAACGGTAGATTTAAATTGTTT</u> ACTAGCCATTTTGTCTTTATCTCC -3'
LF2647R2	5'- TGACATGACTATCCTGACTTGC -3'
RF2651F2	5'- AACGTTCAACGGCAGATAAGCC -3'
LF423F	5' -AATTGATACATGTGGTTTCGAAAG-3'
LF423R	5'- <u>CGAACGGTAGATTTAAATTGTTT</u> CCAATGCATACTTGTACTCCC -3'
RF423F	5' - <u>GTACAGCCCGGGCATGAG</u> CGACTTGATCAATAGCTGAGGG-3'

RF423R	5' -TTGGTTGCCTTGATCGTGTAAG-3'
LF423F2	5' -CTTCAGTTATCGCTACAATCAACG-3'
RF423R2	5' -ACTAACGTACTTTGCACCACGG-3'
LF419F	5'- <u>GTACAGCCCCGGGCATGAG</u> GACGAGTAATCATCCATTCTGA-3'
LF419R	5' -ATGAGTTTGCAATGGAGCTTAGG-3'
RF422F	5' -CAAAGACGTGCCGAATATAGCC-3'
RF422R	<u>5'- CGAACGGTAGATTTAAATTGTTT</u> AAACTGTAGCATAAATAATCCCC-3'
LF419R2	5' -GAGATAATTATTGTAAGACCGTC-3'
RF422F2	5'-CTAACGCATCAATAATCTTACTGG-3'
LF2991F	5'- CCGTTTACTGAACGACTTGTCG-3'
LF2991R	5'- <u>CGAACGGTAGATTTAAATTGTTT</u> TGAAAAATTCATTTTCACACCTCC -3'
RF2991F	5'- <u>GTACAGCCCCGGGCATGAG</u> AAGACTTCAGATTAGGTGTTTCAG -3'
RF2991R	5'- TACTCGTCATTCTAACTACCGC-3'
LF2991F2	5'-TGGCACCGATAATCCCTAAAGC-3'
RF2991R2	5'-TGTAATCTTAATCCGCTTTCACAC-3'
LF0423F	5'-AATTGATACATGTGGTTTCGAAAG-3'
LF0423R	5'- <u>CGAACGGTAGATTTAAATTGTTT</u> CCAATGCATACTTGTACTCCC -3'
RF0429F	5'- <u>GTACAGCCCCGGGCATGAG</u> TTGGTTCCTAGCTAAAATAGGGG-3'
RF0429R	5'- TTTACGATTGAACATCAGGTACG-3'
LF0423R2	5'-CTTCAGTTATCGCTACAATCAACG-3'
RF0429F2	5'- TAATTGCCCAATTGGACCCGAC-3'

TABLE 3. Candidate genes for immunomodulation

Gene name	Gene nr	product	Predicted cytokine stimulation ^a
lp_1953	lp_1953	Hypothetical protein	IL10 1.6x ↑
pts19ADCB	lp_2647-2651	N-galactosamine PTS, EIIADCB transcription regulator, GntR family	IL10 1.7x ↓
plnIFE	lp_0419-0422	Immunity protein plnI Bacteriocin like peptide plnF Bacteriocin like peptide pln E	IL10/IL12 1.7x↓
plnG	lp_0423	ABC transporter	IL10/IL12 1.8x↓
lamB	lp_3582	accessory gene regulator protein B	IL10/IL12 1.3x↓
Prophage P2b protein 1 and 21	lp_2460	prophage P2b protein 21	IL10/IL12 1.5x↑
	lp_2480	prophage P2b protein 1, integrase	

^a Phenotype in PBMC assay affected by the presence of the gene and the magnitude of the effect. ↑ indicates a higher effect when the gene is present, ↓ indicates a lower effect.

5 **TABLE 4.** Candidate genes identified in *in silico* analysis using DCs

Gene name	Gene nr	Description	Predicted phenotype ^a	k.o.
lp_2991	lp_2991	Transcription regulator	IL-10 and TNF-alpha ↑	
plnEFI	lp_0419-lp_0422	Bacteriocin like peptide E	IL-10 ↑	
		Bacteriocin like peptide F		
		Immunity protein plnI		
plnG	lp_0423	ABC transporter	IL-10 ↑	
plnGHSTUVWX	lp_0423-30	Bacteriocin ABC-transporter, ATP-binding and permease protein plnG	IL-10 ↑	
bsh1	lp_3536	choloylglycine hydrolase	TNF-alpha ↑	

^aPhenotype in DC assay affected by the presence of the gene.

↑ indicated a higher effect when the gene is absent

CLAIMS

1. A method for preparing a bacterium capable of immunomodulation, said method comprising the step of knocking out or incorporating one or more genes encoding:
 - 5 a) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
 - b) a polypeptide of a N-acetyl-galactosamine phosphotransferase system,
 - c) a polypeptide encoded by a *lamBDCA* gene cluster,
 - d) a polypeptide encoded by a bacteriocin gene cluster, and/or
 - 10 e) a polypeptide encoded by a bacteriocin transport gene cluster,in said bacterium.
2. A method according to claim 1, said bacterium having anti-inflammatory capacities, said method comprising the step of knocking out one or more genes
15 encoding:
 - i) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
 - ii) a polypeptide of a N-acetyl-galactosamine phosphotransferase system,
 - iii) a polypeptide encoded by a *lamBDCA* gene cluster,
 - 20 iv) a polypeptide encoded by a bacteriocin gene cluster, and/or
 - v) a polypeptide encoded by a bacteriocin transport gene cluster,in said bacterium.
3. A method according to claim 1, said bacterium having pro-inflammatory
25 capacities, said method comprising the step of incorporating one or more genes encoding:
 - i) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
 - ii) a polypeptide of a N-acetyl-galactosamine phosphotransferase system,
 - 30 iii) a polypeptide encoded by a *lamBDCA* gene cluster,
 - iv) a polypeptide encoded by a bacteriocin gene cluster, and/or
 - v) a polypeptide encoded by a bacteriocin transport gene cluster,in said bacterium.

4. A method for modulating expression of one or more genes encoding:
- a) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
 - 5 b) a polypeptide of a N-acetyl-galactosamine phosphotransferase system,
 - c) a polypeptide encoded by a *lamBDCA* gene cluster,
 - d) a polypeptide encoded by a bacteriocin gene cluster, and/or
 - e) a polypeptide encoded by a bacteriocin transport gene cluster,
- 10 said method comprising the step of selecting fermentation conditions resulting in reduced expression of one or more polypeptides according to any one of a), b) and c) compared to standard fermentation conditions.
5. A method for identifying a bacterium capable of immunomodulation, said method comprising the step of detecting the presence or absence of expression of one or more
- 15 genes encoding:
- a) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
 - b) a polypeptide of a N-acetyl-galactosamine phosphotransferase system,
 - c) a polypeptide encoded by a *lamBDCA* gene cluster,
 - 20 d) a polypeptide encoded by a bacteriocin gene cluster, and/or
 - e) a polypeptide encoded by a bacteriocin transport gene cluster.
6. Method according to any of the preceding claims, wherein the polypeptide of a N-acetylgalactosamine phosphotransferase system is selected from SEQ ID NO.s 1, 2,
- 25 3, 4 or 5, or homologues thereof having at least 25% identity therewith.
7. Method according to any of the preceding claims, wherein the polypeptide encoded by a *lamBDCA* gene cluster is selected from SEQ ID NO.s 16, 17, 18, or 19, or homologues thereof having at least 25% identity therewith.
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8. Method according to any of the preceding claims, wherein the polypeptide encoded by a bacteriocin gene cluster is selected from SEQ ID Nos 6, 7, 8 or 9, or homologues thereof having at least 25% identity therewith.

9. Method according to any of the preceding claims, wherein the polypeptide encoded by a bacteriocin transport gene cluster is selected from SEQ ID Nos:10, 11, 12, 13, 14, and/or 15, or homologues thereof having at least 25% identity therewith.

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10. Method according to any of the preceding claims, wherein the bacterium is a Gram-positive bacterium, preferably a lactic acid bacterium, more preferably belonging to the genus *Lactobacillus*.

10 11. A recombinant bacterium obtainable by the method according to any of claims 1-3.

12. Composition comprising a recombinant bacterium as defined in claim 11 and a pharmaceutically or physiologically acceptable carrier.

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13. Food composition comprising a recombinant bacterium as defined in claim 11.

14. A recombinant bacterium as defined in claim 11 or a composition as defined in claim 12 or 13 for use as a medicament.

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15. A recombinant bacterium as defined in claim 11 or a composition as defined in claim 12 or 13, or a bacterium lacking a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, a bacterium lacking a polypeptide of a N-acetylgalactosamine phosphotransferase system, a bacterium
25 lacking a polypeptide encoded by a lamBDCA gene cluster, a bacterium lacking a polypeptide encoded by a bacteriocin gene cluster, and/or a bacterium lacking a polypeptide encoded by a bacteriocin transport gene cluster, for use as a medicament for treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis.

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16. Use of a recombinant bacterium obtainable by the method according to claim 2, or a bacterium lacking a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, a bacterium lacking a polypeptide of a

N-acetylgalactosamine phosphotransferase system, a bacterium lacking a polypeptide encoded by a *lamBDCA* gene cluster, a bacterium lacking a polypeptide encoded by a bacteriocin gene cluster, and/or a bacterium lacking a polypeptide encoded by a bacteriocin transport gene cluster, in the manufacture of a medicament for treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis.

17. A polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith, a polypeptide of a N-acetylgalactosamine phosphotransferase system, a polypeptide encoded by a *lamBDCA* gene cluster, or a polypeptide encoded by a bacteriocin gene cluster, and/or a polypeptide encoded by a bacteriocin transport gene cluster, for use as a medicament.

18. A polypeptide according to claim 17 for use as a medicament for prevention and/or treatment of inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and/or for stimulation of the immune system.

19. A nucleic acid construct comprising one or more genes encoding:

- a) a polypeptide encoded by SEQ ID NO:20, or homologues thereof having at least 25% identity therewith,
- b) a polypeptide of a N-acetylgalactosamine phosphotransferase system,
- c) a polypeptide encoded by a *lamBDCA* gene cluster,
- d) a polypeptide encoded by a bacteriocin gene cluster, and/or
- e) a polypeptide encoded by a bacteriocin transport gene cluster,

operably linked to a promoter, for use as a medicament.

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20. A nucleic acid construct according to claim 19 for use as a medicament for prevention and/or treatment of inflammatory bowel disease such as Crohn disease and ulcerative colitis, and/or for stimulation of the immune system.

21. Use of a polypeptide as defined in claim 17 or a nucleic acid construct as defined in claim 19 in the preparation of a medicament for prevention and/or treatment of inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and/or for stimulation of the immune system.