



(51) International Patent Classification:

A23C 9/123 (2006.01) C12R 1/225 (2006.01)

(21) International Application Number:

PCT/EP2016/070398

(22) International Filing Date:

30 August 2016 (30.08.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

15183194.8 31 August 2015 (31.08.2015) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

## Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

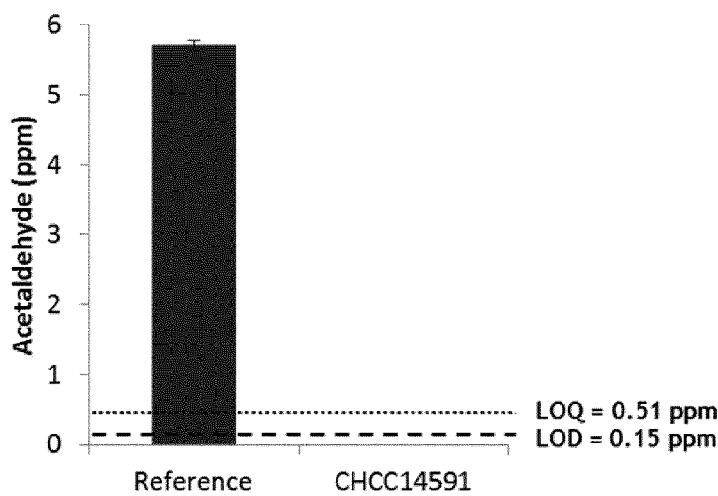
## Published:

— with international search report (Art. 21(3))

— with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))

(54) Title: LACTOBACILLUS FERMENTUM BACTERIA INHIBITING POST-ACIDIFICATION

Figure 4



(57) **Abstract:** The present invention relates to a bacterium of the species *Lactobacillus fermentum*, wherein the bacterium is characterized in that it increases the pH of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*. In a related embodiment the present invention provides a bacterium of the species *Lactobacillus fermentum* characterized in that a fermented milk product comprising the *Lactobacillus fermentum* maintains a pH above 4.0 when stored for at least 14 days at 25 °C, wherein the fermented milk product is obtained by a method comprising incubating a milk with the *Lactobacillus fermentum* in a concentration of at least 10<sup>7</sup> CFU/g and with a starter culture, fermenting until a pH of 4.6 is reached, shaking the fermented product and cooling. The invention further relates to compositions comprising the bacteria, methods for producing fermented milk products using the bacteria and the products thus obtained.

**LACTOBACILLUS FERMENTUM BACTERIA INHIBITING POST-ACIDIFICATION****FIELD OF THE INVENTION**

The present invention relates *Lactobacillus fermentum* bacteria which inhibit post-acidification, an effect frequently observed in fermented milk products stored at temperatures at and above refrigeration temperature. The invention further provides adjunct cultures comprising the bacteria, method of producing a fermented milk product using the bacteria or the cultures and the fermented milk products thus obtained, including food, feed and pharmaceutical products.

10

**BACKGROUND OF THE INVENTION**

Lactic acid bacteria (LAB) have been used over decades for increasing the shelf life of food products. During fermentation LAB produce lactic acids as well as other organic acids which cause a reduction of pH of the fermented product. Products having an acidic pH do not support further growth of most microorganisms, including pathogenic and spoilage organisms. However, growth of yeasts and molds is not affected by low pH and often causes spoilage of fermented dairy products.

Traditionally, yoghurt is produced by fermentation of milk with a specific yoghurt starter culture consisting of a mixture of two species of lactic acid bacteria (LAB), *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The main role of the starter in the production of yoghurt are (i) acidification through the conversion of lactose into lactic acid, (ii) creation of viscous texture e.g. by denaturation of proteins and production of exopolysaccharides, and (iii) development of the typical yoghurt flavor (1).

25 The typical yoghurt flavor is caused by lactic acid, which imparts an acidic and refreshing taste, and a mixture of various carbonyl compounds like acetone, diacetyl, and acetaldehyde, the latter of which is considered the major flavor component (2).

After termination of fermentation the yoghurt is stored until consumed. During this period, 30 the LAB may cause further acidification of the product, in particular when stored above refrigeration temperatures. This phenomenon is called post-acidification and is generally considered to represent an undesired trait observed in most yoghurt types over time. Post-acidification may contribute to defects such as syneresis, increased reduction of viable counts, accumulation of lactic acid in the product and development of an undesired flavor. It is mainly 35 due to the uncontrollable growth of strains of *L. delbrueckii* subsp. *bulgaricus* at low pH values and refrigerated temperatures (4). It can be influenced to a limited extent by applying good manufacturing practice and by using cultures with reduced post-acidification behavior, as e.g. Chr. Hansen Mild 2.0®. Another possibility to circumvent this problem is to pasteurize the

yogurt after its manufacture. Although this manipulation would aid the manufacturers considerably in handling and distribution, yogurt pasteurization is not commonly applied because many consumers wish to obtain fermented milk products containing live microorganisms providing an intact lactase activity. Further, mildly-acidifying yogurt-related cultures containing *L. acidophilus* and bifidobacteria offer the advantage of less post-acidification.

At the same time, it has been observed that starter cultures with antifungal effects, i.e. cultures also known as bioprotective bacterial starter cultures (e.g. Holdbac® YM-C plus and FreshQ®4), appear to increase post-acidification in methods of producing fermented milk products, in particular when the fermented products are stored at ambient temperatures.

As a consequence of these findings, there is still a need to improve the control of post-acidification in methods for producing fermented milk products.

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#### **SUMMARY OF THE INVENTION**

The present invention provides a bacterium of the species *Lactobacillus fermentum* characterized in that it increases the pH (i.e. counteracts the postacidification) of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*. The increase in pH can be any increase, but preferably reaches a value of at least 0.1. The increase is preferably determined after storing the fermented product over 21 days at 25 °C.

25 The inventors surprisingly found that the *Lactobacillus fermentum* strains of the present invention not only do not contribute to post-acidification, but actually antagonize post-acidification by increasing the pH value of a fermented milk product produced with these strains. As is shown in the Examples, below, the effect is particularly notable in fermentation processes using starter cultures exhibiting significant post-acidification, including a number of 30 commercially available starter cultures and in particular when used together with commercial antifungal bacteria. A total of 10 different *Lactobacillus fermentum* strains were identified that inhibit post-acidification of starter cultures.

In a related embodiment the present invention also provides a bacterium of the species 35 *Lactobacillus fermentum* characterized in that a fermented milk product comprising the *Lactobacillus fermentum* maintains a pH above 4.0 when stored for at least 14 days at 25 °C, wherein the fermented milk product is obtained by a method comprising incubating a milk

with the *Lactobacillus fermentum* in a concentration of at least  $10^7$  CFU/g and with a starter culture, fermenting until a pH of 4.6 is reached, shaking the fermented product and cooling.

The bacteria of the present invention may additionally be characterized in reducing the presence of acetaldehyde as produced by other bacteria in the starter culture. For example, certain *Lactobacillus fermentum* strains of the present invention may further be characterized in having the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50%.

In a related embodiment the *Lactobacillus fermentum* strains of the present invention can further be characterized in that the bacterium secretes diacetyl in a range of 0 to 5 ppm.

The present invention therefore provides the bacteria as described above, compositions comprising the same, methods using the bacteria for producing fermented milk products, as well as the products thus obtained.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a bacterium of the species *Lactobacillus fermentum* characterized in that it increases the pH of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*. The increase in pH can be any increase, but preferably reaches a value of at least 0.1. The increase is preferably determined after storing the fermented product over 21 days at 25 °C.

The *Lactobacillus fermentum* strains of the present invention can for example be characterized in that they increase the pH (i.e. counteracts the postacidification) of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*, wherein the increase in pH is at least by a value of 0.1 and is determined after storing the fermented product over 21 days at 25 °C, and wherein the starter culture used for the preparation of the fermented milk product comprises LAB which are able to decrease the pH of a milk product during fermentation to a value of pH 4.6 in 10 hours or less. For example, the assay may be based on mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Respective mixtures are frequently used for the production of yoghurt and known to cause post-acidification.

In an alternative aspect the present invention also provides a bacterium of the species *Lactobacillus fermentum* characterized in that a fermented milk product comprising the

5 *Lactobacillus fermentum* maintains a pH above 4.0 when stored for at least 14 days at 25 °C, wherein the fermented milk product is obtained by a method comprising incubating a milk with the *Lactobacillus fermentum* in a concentration of at least 10<sup>7</sup> CFU/g and with a starter culture, fermenting until a pH of 4.6 is reached, shaking the fermented product and cooling. It should be understood that the feature specifying that the *Lactobacillus fermentum* strains of the present invention can maintain the pH above 4.0 when stored for at least 14 days at 25 °C merely characterizes the assay generally used to determine the effect. It is not necessary or required that the *Lactobacillus fermentum* strains of the present invention, compositions comprising the same, including food or feed products are in fact stored under these conditions. Again, in one aspect the assay can be carried out using a starter culture comprising LAB which are able to decrease the pH of a milk product during fermentation to a value of pH 4.6 in 10 hours or less. For example, the assay may be based on mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

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15 The *Lactobacillus fermentum* strains of the present invention have particular advantages as they reduce the risk of post-acidification and thus improve the storage stability of food products made with these bacteria, in particular the storage stability under conditions above refrigeration temperatures.

20 As indicated in the Examples, the effect of the *Lactobacillus fermentum* strains of the present invention of increasing the pH of a fermented milk product was observed when the same were added to the milk prior to fermentation in a concentration of least 10<sup>7</sup> CFU/g. The invention therefore encompasses the *Lactobacillus fermentum* strains of the present invention in a concentration of at least 10<sup>7</sup> CFU/g, compositions comprising the same and fermented food products comprising the same. Preferred concentration ranges include a concentration of 10<sup>7</sup> CFU/g to 10<sup>11</sup> CFU/g, 10<sup>7</sup> CFU/g to 10<sup>10</sup> CFU/g and 10<sup>7</sup> CFU/g to 10<sup>9</sup> CFU/g.

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30 Common starter cultures, such as commercially available mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are known to produce volatile compounds which contribute significantly to the sensory properties of the fermented products. According to one aspect the bacteria of the invention are further characterized in secreting low amounts or essentially no volatile compounds, which affect the sensory properties of the food product. Acetaldehyde, diacetyl and acetoin are known volatile compounds, which affect the sensory properties of the food product.

35 In a related embodiment the bacteria of the present invention are characterized in reducing the presence of acetaldehyde as produced by other bacteria in the starter culture. For example, certain *Lactobacillus fermentum* strains of the present invention may further be

characterized in having the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50%. Different assays are known in the art for determining the concentration of acetaldehyde in a fermented product and can be used for that purpose in accordance with the present invention. The ability 5 to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50% is preferably determined in an assay comprising:

- (1) preparing a fermented milk product by:
  - (a) incubating a milk with the *Lactobacillus fermentum* in a concentration of at least 10<sup>7</sup> CFU/g and with a starter culture,
  - (b) fermenting until a pH of 4.6 is reached, and;
- (2) storing the fermented milk product at 7±1°C for 14 days;
- (3) adding 200 µl of 4N H<sub>2</sub>SO<sub>4</sub> to 1 g of the fermented milk product and determining the concentration of acetaldehyde by static head space gas chromatography.

15

Acetaldehyde is a taste component produced by lactic acid bacteria during fermentation. While the component is desirable in certain applications, it would be advantageous to reduce or avoid the presence of acetaldehyde in other applications. *Lactobacillus fermentum* bacteria reducing the concentration of acetaldehyde in a fermented milk product therefore provide 20 advantages in specific applications, for example when preparing sweetened yoghurt. The *Lactobacillus fermentum* strains of the present invention may for example reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 75%, at least 95% or at least 98%.

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Alternatively or additionally, the *Lactobacillus fermentum* strains of the present invention can be characterized in that the bacterium secretes diacetyl in a range of 0 to 5 ppm. Again, different assays are known in the art for determining the concentration of diacetyl in a fermented product and can be used for that purpose in accordance with the present invention. The ability to reduce the concentration of diacetyl produced by a starter culture during 30 fermentation in a fermented milk product by at least 50% is preferably determined in an assay comprising:

- (1) preparing a fermented milk product by:
  - (a) incubating a milk with the *Lactobacillus fermentum* in a concentration of at least 35 10<sup>7</sup> CFU/g and with a starter culture,
  - (b) fermenting until a pH of 4.6 is reached, and;
- (2) storing the fermented milk product at 7±1°C for 14 days;

(3) adding 200 µl of 4N H<sub>2</sub>SO<sub>4</sub> to 1 g of the fermented milk product and determining the concentration of diacetyl by static head space gas chromatography.

*Lactobacillus fermentum* bacteria secreting low concentrations of diacetyl have advantages in methods of producing food products with these bacteria, as the strains that produce high concentrations of these volatile compounds affect the taste of the final product and can therefore not be used for all applications. *Lactobacillus fermentum* strains of the present invention can be characterized in that the bacterium secretes diacetyl in a range of 0 to 3 ppm or 0 to 2ppm.

10

Bacteria of the present invention may advantageously be derived from one of the following deposited strains:

(a) the *Lactobacillus fermentum* strain CHCC12798 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32084;

(b) the *Lactobacillus fermentum* strain CHCC12797 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32085;

(c) the *Lactobacillus fermentum* strain CHCC14591 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32086;

(d) the *Lactobacillus fermentum* strain CHCC14588 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32087;

(e) the *Lactobacillus fermentum* strain CHCC15844 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32088;

(f) the *Lactobacillus fermentum* strain CHCC15865 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32089;

(g) the *Lactobacillus fermentum* strain CHCC15847 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32090;

5 (h) the *Lactobacillus fermentum* strain CHCC15848 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32091;

10 (i) the *Lactobacillus fermentum* strain CHCC15926 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 32096;

15 (j) the *Lactobacillus fermentum* strain CHCC2008 as deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig under the accession No.: 22584;

20 (k) a mutant strain obtainable from one the deposited bacteria according to (a) to (j), wherein the mutant is characterized in that it increases the pH of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*.

The increase in pH caused by the mutant can be any increase, but preferably reaches a value of at least 0.1. The increase is preferably determined after storing the fermented product over 25 21 days at 25 °C.

In the context of the present application, the term "lactic acid bacteria" or "LAB" is used to refer to food-grade bacteria producing lactic acid as the major metabolic end-product of carbohydrate fermentation. These bacteria are related by their common metabolic and 30 physiological characteristics and are usually Gram positive, low-GC, acid tolerant, non-sporulating, non-respiring, rod-shaped bacilli or cocci. During the fermentation stage, the consumption of lactose by these bacteria causes the formation of lactic acid, reducing the pH and leading to the formation of a protein coagulum. These bacteria are thus responsible for the acidification of milk and for the texture of the dairy product. As used herein, the term 35 "lactic acid bacteria" encompasses, but is not limited to, bacteria belonging to the genus of *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., *Lactococcus* spp., such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus lactis*, *Bifidobacterium animalis*, *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*,

*Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Bifidobacterium breve* and *Leuconostoc* spp.

Depending on the optimum temperature for propagation, LAB are characterized as mesophilic or thermophilic LAB. The term "mesophile" refers to microorganisms that thrive best at moderate temperatures. The term "mesophilic fermentation" herein refers to fermentation at a temperature between about 22°C and about 35°C. The term "mesophilic fermented milk product" refers to fermented milk products prepared by mesophilic fermentation of a mesophilic starter culture and include such fermented milk products as buttermilk, sour milk, cultured milk, smetana, sour cream and fresh cheese, such as quark, tvarog and cream cheese. The industrially most useful mesophilic bacteria include *Lactococcus* spp. and *Leuconostoc* spp.

The term "thermophile" refers to microorganisms that thrive best at high temperatures. The term "thermophilic fermentation" refers to fermentation methods carried out at a temperature between about 35°C and about 45°C. The term "thermophilic fermented milk product" refers to fermented milk products prepared by thermophilic fermentation using a thermophilic starter culture and include such fermented milk products as set-yoghurt, stirred-yoghurt, strained yoghurt and drinking yoghurt. The industrially most useful thermophilic bacteria include *Streptococcus* spp. and *Lactobacillus* spp.

As will be outlined below, the present invention encompasses methods using mesophilic and thermophilic fermentation.

The terms "inhibit" in relation to fungi, yeasts and molds refers to a decrease in the growth or sporulation or a reduction in the number or in the concentration of fungi, yeasts and molds, for example in food products and/or on the surface of food products comprising the bacteria of the present invention in relation to food products which do not comprise such bacteria. The extent of inhibition provided by the *Lactobacillus fermentum* bacteria of the present invention is preferably determined by growth on agar solidified fermented milk in the presence and absence of the *Lactobacillus fermentum* bacteria.

In the present context, the term "mutant" should be understood as a strain derived from a strain of the invention by means of e.g. genetic engineering, radiation and/or chemical treatment. It is preferred that the mutant is a functionally equivalent mutant, e.g. a mutant that has substantially the same, or improved, properties in particular in relation to the effects on inhibiting post-acidification, as the deposited strain. Such a mutant is a part of the present invention. Especially, the term "mutant" refers to a strain obtained by subjecting a strain of

the invention to any conventionally used mutagenization treatment including treatment with a chemical mutagen such as ethane methane sulphonate (EMS) or N-methyl-N'-nitro-N-nitroguanidine (NTG), UV light or to a spontaneously occurring mutant. A mutant may have been subjected to several mutagenization treatments (a single treatment should be

5 understood one mutagenization step followed by a screening/selection step), but it is presently preferred that no more than 20, or no more than 10, or no more than 5, treatments (or screening/selection steps) are carried out. In a presently preferred mutant, less than 5%, or less than 1% or even less than 0.1% of the nucleotides in the bacterial genome have been shifted with another nucleotide, or deleted, compared to the mother strain.

10

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

15

The present invention further provides compositions comprising at least one bacterium of the species *Lactobacillus fermentum* characterized in that a fermented milk product comprising the *Lactobacillus fermentum* maintains a pH above 4.0 when stored for at least 14 days at 25 °C in the assay as described above.

20

Respective compositions may comprise numerous further bacteria including LABs. A preferred composition of the present invention is therefore characterized in that the composition further comprises at least one further bacterium selected from one or more of the following genera and species *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., *Lactococcus* spp.,

25 such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus lactis*, *Bifidobacterium animalis*, *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Bifidobacterium breve* and *Leuconostoc* spp.

30

In a particularly preferred embodiment, the compositions of the present invention comprise at least one bacterium of the species *Lactobacillus fermentum* inhibiting post-acidification as described above and one or more second bacterium. In one embodiment, several different strains of the *Lactobacillus fermentum* bacteria of the present invention are combined. The further bacteria can for example be selected from bacteria with antifungal activities:

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(a) *Lactobacillus rhamnosus* bacterium of strain CHCC15860 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM32092;

(b) *Lactobacillus rhamnosus* bacterium of strain CHCC5366 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM23035;

5 (c) *Lactobacillus rhamnosus* bacterium of strain CHCC12697 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24616;

(d) *Lactobacillus paracasei* bacterium of strain CHCC12777 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24651; and

10 (e) *Lactobacillus paracasei* bacterium of strain CHCC14676 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM25612.

15 The compositions of the present invention may in addition comprise numerous further components, including one or more cryoprotective compounds as well as flavoring compounds.

20 LAB are most commonly added in the form of a starter culture to milk. The term "starter" or "starter culture" as used in the present context refers to a culture of one or more food-grade micro-organisms, in particular lactic acid bacteria, which are responsible for the acidification 25 of the milk base. Starter cultures may be fresh, but are most frequently frozen or freeze-dried. These products are also known as "Direct Vat Set" (DVS) cultures and are produced for direct inoculation of a fermentation vessel or vat for the production of a dairy product, such as a fermented milk product or a cheese. Respective starter cultures are commercially available 30 from numerous sources and include F-DVS YoFlex Mild 2.0, F-DVS YF-L901, FD-DVS YF-812 and F-DVS CH-1, four cultures commercially available from Chr. Hansen containing mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

35 In one aspect the present invention therefore provides compositions in the form of a solid frozen or freeze-dried starter culture comprising lactic acid bacteria in a concentration of at least  $10^9$  colony forming units per g of frozen material or in a concentration of at least  $10^{10}$  colony forming units per g of frozen material or in a concentration of at least  $10^{11}$  colony forming units per g of frozen material which compositions include a bacterium of the species *Lactobacillus fermentum* which inhibits post-acidification as described above.

The present invention further provides *Lactobacillus fermentum* bacteria and compositions comprising the same which are characterized by more than one of the above features. For

example, the present invention provides a bacterium of the species *Lactobacillus fermentum*, wherein the bacterium is characterized in that it increases the pH of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*,

5 wherein the increase in pH is at least by a value of 0.1,

and wherein the increase in pH is determined after storing a product fermented with a starter culture and the *Lactobacillus fermentum* in a concentration of at least 10<sup>7</sup> cfu/g over 21 days at 25 °C, and

10 wherein the bacterium has the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50%.

In a further embodiment the present invention provides methods of producing a fermented milk product which comprise adding the *Lactobacillus fermentum* bacterium which inhibits post-acidification as described above or the composition comprising the same to milk or to a milk product and fermenting the mixture at a temperature between about 22°C and about 43°C until a pH of less than 4.6 is reached.

20 In the context of the present application, the term "milk" is broadly used in its common meaning to refer to liquids produced by the mammary glands of animals or by plants. In accordance with the present invention the milk may have been processed and the term "milk" includes whole milk, skim milk, fat-free milk, low fat milk, full fat milk, lactose-reduced milk, or concentrated milk. Fat-free milk is non-fat or skim milk product. Low-fat milk is typically 25 defined as milk that contains from about 1% to about 2% fat. Full fat milk often contains 2% fat or more. The term "milk" is intended to encompass milks from different mammals and plant sources. Mammal sources of milk include, but are not limited to cow, sheep, goat, buffalo, camel, llama, mare and deer. Plant sources of milk include, but are not limited to, milk extracted from soy bean, pea, peanut, barley, rice, oat, quinoa, almond, cashew, 30 coconut, hazelnut, hemp, sesame seed and sunflower seed. In the methods and products of the present invention, milk derived from cows is most preferably used as a starting material for the fermentation.

The term "milk" also includes fat-reduced and/or lactose-reduced milk products. Respective 35 products can be prepared using methods well known in the art and are commercially available (for example from Select Milk Producers Inc., Texas, USA). Lactose-reduced milk can be produced according to any method known in the art, including hydrolyzing the lactose by

lactase enzyme to glucose and galactose, or by nanofiltration, electrodialysis, ion exchange chromatography and centrifugation.

The term "milk product" or "milk base" is broadly used in the present application to refer to a composition based on milk or milk components which can be used as a medium for growth and fermentation of LAB. The milk product or base comprises components derived from milk and any other component that can be used for the purpose of growing or fermenting LAB.

The fermentation step of the process for manufacturing fermented dairy products comprises the addition of LAB to milk. Fermentation processes used in production of dairy products are well known and a person of ordinary skill can select fermentation process conditions, including temperature, oxygen, amount and characteristics of microorganism(s) and fermentation time.

Prior to fermentation, the milk substrate may be homogenized and pasteurized according to methods known in the art. "Homogenizing" as used herein means intensive mixing to obtain a soluble suspension or emulsion. If homogenization is performed prior to fermentation, it may be performed so as to break up the milk fat into smaller sizes so that it no longer separates from the milk. This may be accomplished by forcing the milk at high pressure through small orifices. "Pasteurizing" as used herein means treatment of the milk substrate to reduce or eliminate the presence of live organisms, such as microorganisms. Preferably, pasteurization is attained by maintaining a specified temperature for a specified period of time. The specified temperature is usually attained by heating. The temperature and duration may be selected in order to kill or inactivate certain bacteria, such as harmful bacteria. A rapid cooling step may follow.

In a particularly advantageous method of the present invention the *Lactobacillus fermentum* bacterium which inhibits post-acidification as described above or the composition comprising the same is added to milk or to a milk product and the mixture is fermented in such a manner that

- (a) the concentration of the *Lactobacillus fermentum* bacteria which inhibits post-acidification is at least  $1 \times 10^6$  cfu/g or at least  $1 \times 10^7$  cfu/g at the termination of fermentation in the fermented milk product; and/or
- (b) such that the concentration of the *Lactobacillus fermentum* bacteria which inhibits post-acidification is at least  $1 \times 10^5$  cfu/cm<sup>2</sup> on the surface of the fermented milk product.

This way of proceeding has the advantage that post-acidification inhibiting effect of the *Lactobacillus fermentum* bacterium can be fully used.

The present invention further provides methods of, wherein the fermented product is stored at a temperature above 7°C, preferably at a temperature between 7°C and 25°C. The product may be stored at any time, but is preferably stored for a period of at least 14 days and wherein the pH of the fermented milk product is maintained above pH 4.0 during storage.

5 The invention further provides methods of producing a food, feed or pharmaceutical product comprising a method of producing a fermented milk product as described above and the food, feed or pharmaceutical product obtainable by this method.

10 Fermentation is carried out to produce food products, feed products or pharmaceuticals. The terms "fermented milk product", "food" or "feed" product refer to products obtainable by the 15 fermentation methods of the present invention and include cheese, yoghurt, fruit yoghurt, yoghurt beverage, strained yoghurt (Greek yoghurt, Labneh), quark, fromage frais and cream cheese. The term food further encompasses other fermented food products, including fermented meat, such as fermented sausages, and fermented fish products.

20 The term "cheese" is understood to encompass any cheese, including hard, semi-hard and soft cheeses, such as cheeses of the following types: Cottage, Feta, Cheddar, Parmesan, Mozzarella, Emmentaler, Danbo, Gouda, Edam, Feta-type, blue cheeses, brine cheeses, Camembert and Brie. The person skilled in the art knows how to convert the coagulum into cheese, methods can be found in the literature, see e.g. Kosikowski, F. V., and V. V. Mistry, 25 "Cheese and Fermented Milk Foods", 1997, 3rd Ed. F. V. Kosikowski, L. L. C. Westport, CT. As used herein, a cheese which has a NaCl concentration below 1.7% (w/w) is referred to as a "low-salt cheese".

30 In the context of the present application, the term "yoghurt" refers to products comprising *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and optionally other microorganisms such as *Lactobacillus delbrueckii* subsp. *lactis*, *Bifidobacterium animalis* subsp. *lactis*, *Lactococcus lactis*, *Lactobacillus acidophilus* and *Lactobacillus paracasei*, or any 35 microorganism derived therefrom. The lactic acid strains other than *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, are included to give the finished product various properties, such as the property of promoting the equilibrium of the flora. As used herein, the term "yoghurt" encompasses set yoghurt, stirred yoghurt, drinking yoghurt, Petit Suisse, heat treated yoghurt, strained or Greek style yoghurt characterized by a high protein level and yoghurt-like products.

In particular, term "yoghurt" encompasses, but is not limited to, yoghurt as defined according to French and European regulations, e.g. coagulated dairy products obtained by lactic acid fermentation by means of specific thermophilic lactic acid bacteria only (i.e. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) which are cultured

5 simultaneously and are found to be live in the final product in an amount of at least 10 million CFU (colony-forming unit) / g. Yoghurts may optionally contain added dairy raw materials (e.g. cream) or other ingredients such as sugar or sweetening agents, one or more flavoring(s), fruit, cereals, or nutritional substances, especially vitamins, minerals and fibers, as well as stabilizers and thickeners. Optionally the yoghurt meets the specifications for  
10 fermented milks and yoghurts of the AFNOR NF 04-600 standard and/or the codex StanA-IIa-1975 standard. In order to satisfy the AFNOR NF 04-600 standard, the product must not have been heated after fermentation and the dairy raw materials must represent a minimum of 70% (m/m) of the finished product.

15 In a further embodiment the present invention provides food, feed or pharmaceutical products comprising one or more bacteria of the species *Lactobacillus fermentum* which inhibits post-acidification as described above and one or more of:

- (a) least one further bacterium selected from one or more of the following genera  
20 *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp.,  
*Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp. and  
*Enterococcus* spp.;
- (b) *Lactobacillus rhamnosus* bacterium of strain CHCC15860 as deposited with the  
25 German Collection of Microorganisms and Cell Cultures (DSMZ) under  
accession No. DSM32092;
- (c) *Lactobacillus rhamnosus* bacterium of strain CHCC5366 as deposited with the  
German Collection of Microorganisms and Cell Cultures (DSMZ) under  
accession No. DSM23035;
- (d) *Lactobacillus rhamnosus* bacterium of strain CHCC12697 as deposited with the  
30 German Collection of Microorganisms and Cell Cultures (DSMZ) under  
accession No. DSM24616;
- (e) *Lactobacillus paracasei* bacterium of strain CHCC12777 as deposited with the  
German Collection of Microorganisms and Cell Cultures (DSMZ) under  
accession No. DSM24651; and
- (f) *Lactobacillus paracasei* bacterium of strain CHCC14676 as deposited with the  
35 German Collection of Microorganisms and Cell Cultures (DSMZ) under  
accession No. DSM25612.

## DESCRIPTION OF THE FIGURES

**Figure 1:** pH development in fermented milk products over time when stored at (A)  $7\pm1^\circ\text{C}$  or (B)  $25\pm1^\circ\text{C}$  for 21 days. The products are fermented with starter culture only (Reference,  $\Delta$ ) or starter culture in combination with FreshQ®4 ( $\diamond$ ), Holdbac® YM-C Plus ( $\circ$ ) or Lb. fermentum CHCC14591 ( $\square$ ).

**Figure 2:** pH development in fermented milk products over time when stored at (A)  $7\pm1^\circ\text{C}$  and (B)  $25\pm1^\circ\text{C}$  for 28 days. The products are fermented with starter culture only, Reference, or starter culture in combination with FreshQ®4, Holdbac®® YM-C Plus or Lb. fermentum strains.

**Figure 3:** Acetaldehyde levels after storage at  $7\pm1^\circ\text{C}$  for 14 days in fermented milk products fermented with starter culture alone (Reference), or starter cultures in combination with Lb. fermentum strains. LOD: Limit of detection. LOQ: Limit of quantification.

**Figure 4:** Acetaldehyde levels after storage at  $7\pm1^\circ\text{C}$  for 14 days in fermented milk products fermented with starter culture alone (Reference), or starter cultures in combination with Lb. fermentum CHCC14591. LOD: Limit of detection. LOQ: Limit of quantification.

**Figure 5:** Acidification curves of four commercial starter cultures, FD-DVS YF-L812, F-DVS YF-L901, F-DVS YoFlex Mild 2.0 and F-DVS CH-1, grown in milk (1% fat and 4.5% protein) at  $43^\circ\text{C}$ .

**Figure 6:** Post-acidification curves of yoghurt fermented with one of four commercial starter cultures, FD-DVS YF-L812, F-DVS YF-L901, F-DVS YoFlex Mild 2.0 and F-DVS CH-1 after storage at  $6^\circ\text{C}$  for up to 43 days.

**Figure 7:** Acetaldehyde levels after storage at  $7\pm1^\circ\text{C}$  for 14 days in fermented milk products fermented with starter culture, FD DVS YF-L812 or F-DVS CH-1, alone (Reference), or starter cultures in combination one of the nine Lb. fermentum strains. LOD: Limit of detection. LOQ: Limit of quantification.

**Example 1:****Analysis of the effect of *Lb. fermentum* CHCC14591 on post-acidification**

Reduced-fat (1.5% w/v) homogenized milk was heat-treated at 90±1°C for 20 min and 5 cooled immediately. A commercial starter culture (F-DVS Mild 2.0) was inoculated at 0.02% (v/w), and the inoculated milk was distributed into 200 ml bottles. One bottle was inoculated with *Lb. fermentum* CHCC14591 in total concentration of 2 x 10<sup>7</sup> CFU/g, two bottles were inoculated with two commercial bioprotective cultures (FreshQ®4 and Holdbac® YM-C Plus) in recommended dosages (100U/T and 20 DCU/100L for FreshQ®4 and Holdbac® YM-C Plus, 10 respectively), and one bottle was used as a reference and only inoculated with the starter culture. All bottles were incubated in a water bath at 43±1°C and fermented at these conditions until pH of 4.60±0.1 was reached. After fermentation, the bottles were vigorously shaken to break the coagulum and cooled on ice.

15 To monitor the effect on post acidification, the four fermented milk samples (starter-only, FreshQ®4, Holdbac® YM-C Plus and *Lb. fermentum* CHCC14591) were stored at 7±1°C and 25±1°C for 21 days and pH was measured on day 1, 7, 14 and 21.

The effect is illustrated in Figure 1, showing that addition of *Lb. fermentum* CHCC14591 20 during milk fermentation result in lower post acidification compared to the use of the starter culture alone and particularly compared to the use of the two commercial bioprotective cultures both contributing to post-acidification.

**Example 2:****Analysis of the effect of ten *Lb. fermentum* strains on post-acidification**

Reduced-fat (1.5% w/v) homogenized milk was heat-treated at 90±1°C for 20 min and cooled immediately. A commercial starter culture (F-DVS YF-L901) was inoculated at 0.02% (v/w), and the inoculated milk was distributed into 200 ml bottles. Ten bottles were 30 inoculated with the *Lb. fermentum* strains in concentrations of 1 x 10<sup>7</sup> CFU/g and one bottle was used as a reference and only inoculated with the starter culture. All bottles were incubated in a water bath at 43±1°C and fermented at these conditions until pH of 4.60±0.1 was reached. After fermentation, the bottles were vigorously shaken to break the coagulum and cooled on ice.

35

The tested *Lb. fermentum* strains were: *Lb. fermentum* CHCC12798, *Lb. fermentum* CHCC12797, *Lb. fermentum* CHCC14591, *Lb. fermentum* CHCC14588, *Lb. fermentum*

CHCC15844, *Lb. fermentum* CHCC15865, *Lb. fermentum* CHCC15847, *Lb. fermentum* CHCC15848, *Lb. fermentum* CHCC15926, and *Lb. fermentum* CHCC2008.

To monitor the effect on post acidification, the eleven fermented milk samples (starter culture alone and starter culture in combination with the ten *Lb. fermentum* strains) were stored at 5  $7\pm1^\circ\text{C}$  and  $25\pm1^\circ\text{C}$  for 28 days and pH was measured on day 1, 7, 14, 21 and 28.

The effects on post-acidification are illustrated in Figure 2 and show that each of the *Lb. fermentum* strains *Lb. fermentum* CHCC12798, *Lb. fermentum* CHCC12797, *Lb. fermentum* CHCC14591, *Lb. fermentum* CHCC14588, *Lb. fermentum* CHCC15844, *Lb. fermentum* CHCC15865, *Lb. fermentum* CHCC15847, *Lb. fermentum* CHCC15848, *Lb. fermentum* CHCC15926, and *Lb. fermentum* CHCC2008 does not contribute to post-acidification or even reduce post-acidification compared to reference yoghurt.

15 These findings were unexpected and are highly significant, as prior art antifungal food-grade bacteria were observed to increase the post-acidification effects caused by the starter culture.

**Example 3:**

20 **Effect of the ten *Lb. fermentum* strains on acetaldehyde content**

Ten *Lb. fermentum* strains were tested for their ability to lower acetaldehyde content.

Reduced-fat (1.5% w/v) homogenized milk was heat-treated at  $90\pm1^\circ\text{C}$  for 20 min and 25 cooled immediately. A commercial starter culture (F-DVS YF-L901 Yo-Flex®) was inoculated at 0.02% (v/w), and the inoculated milk was distributed into 200 ml bottles. Ten bottles were inoculated with the *Lb. fermentum* strains in concentrations of  $1 \times 10^7$  CFU/g and one bottle was used as a reference and only inoculated with the starter culture. All bottles were incubated in a water bath at  $43\pm1^\circ\text{C}$  and fermented at these conditions until pH of  $4.60\pm0.1$  30 was reached. After fermentation, the bottles were vigorously shaken to break the coagulum and cooled on ice. The bottles were stored at  $7\pm1^\circ\text{C}$  for 14 days.

On day 14 samples were analyzed for acetaldehyde by static head space gas chromatography (HSGC), a sensitive method for analyzing volatiles in complex matrices. The setup consisted 35 of a Static Head Space sampler connected to Gas Chromatograph with Flame Ionization Detector (FID). For that purpose the following equipment was used:

HS-autosampler: HS40XI, TurboMatrix 110, Perkin Elmer.

HS-software: HSControl v.2.00, Perkin Elmer.

GC: Autosystem XL, Perkin Elmer.

GC-software: Turbochrom navigator, Perkin Elmer.

Column: HP-FFAP 25 m x 0.20 mm x 0.33 µm, Agilent Technologies

5

Standards of known concentration were used to determine response factors (calibration), controls were used to control that the used response factors were stable within an analytical series as well as in-between series and over time (months). Concentration of volatiles (ppm) in samples and controls was determined using response factors coming from standards.

10 Samples were prepared by adding 200 µl of 4N H<sub>2</sub>SO<sub>4</sub> to 1 g yoghurt sample and immediately analyzed by HSGC.

The results are illustrated in Figure 3 and show that each of the strains *Lb. fermentum* CHCC12798, *Lb. fermentum* CHCC12797, *Lb. fermentum* CHCC14591, *Lb. fermentum* CHCC14588, *Lb. fermentum* CHCC15844, *Lb. fermentum* CHCC15865, *Lb. fermentum* CHCC15847, *Lb. fermentum* CHCC15848, *Lb. fermentum* CHCC15926, and *Lb. fermentum* CHCC2008 has the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product.

20 **Example 4:**

**Effect of one *Lb. fermentum* strain on acetaldehyde content**

One *Lb. fermentum* strain was tested for the ability to lower acetaldehyde content.

25 Reduced-fat (1.5% w/v) homogenized milk was heat-treated at 90±1°C for 20 min and cooled immediately. A commercial starter culture (F-DVS Mild 2.0) was inoculated at 0.02% (v/w), and the inoculated milk was distributed into two 200 ml bottles. One bottle was inoculated with the *Lb. fermentum* strains in concentrations of 1 x 10<sup>7</sup> CFU/g and one bottle was used as a reference and only inoculated with the starter culture. Both bottles were 30 incubated in a water bath at 43±1°C and fermented at these conditions until pH of 4.60±0.1 was reached. After fermentation, the bottles were vigorously shaken to break the coagulum and cooled on ice. The bottles were stored at 7±1°C for 14 days.

35 On day 14 samples were analyzed for acetaldehyde by static head space gas chromatography (HSGC), a sensitive method for analyzing volatiles in complex matrices. The setup consisted of a Static Head Space sampler connected to Gas Chromatograph with Flame Ionization Detector (FID). For that purpose the following equipment was used:

HS-autosampler: HS40XI, TurboMatrix 110, Perkin Elmer.

HS-software: HSControl v.2.00, Perkin Elmer.

GC: Autosystem XL, Perkin Elmer.

GC-software: Turbochrom navigator, Perkin Elmer.

5 Column: HP-FFAP 25 m x 0.20 mm x 0.33 µm, Agilent Technologies

Standards of known concentration were used to determine response factors (calibration), controls were used to control that the used response factors were stable within an analytical series as well as in-between series and over time (months). Concentration of volatiles (ppm) 10 in samples and controls was determined using response factors coming from standards. Samples were prepared by adding 200 µl of 4N H<sub>2</sub>SO<sub>4</sub> to 1 g yoghurt sample and immediately analyzed by HSGC.

The results are illustrated in Figure 4 and show that *Lb. fermentum* CHCC14591 has the 15 ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product.

**Example 5:**

**Functional analysis of commercial starter cultures**

20 The three commercial starter cultures included herein were chosen based on their different acidification profiles. Three were frozen, F-DVS CH-1, F-DVS YoFlex Mild 2.0 and F-DVS YF-L901, and one was freeze dried, FD-DVS YF-L812. To test the difference in acidification profiles, semi fat milk was standardized to 1% fat and 4.5% protein with skim milk powder 25 and heat-treated at 85±1°C for 30 min and cooled immediately. One of four different commercial starter cultures (F-DVS CH-1, F-DVS YoFlex Mild 2.0, F-DVS YF-L901 or FD-DVS YF-L812) was inoculated at 0.02% (v/w), and the inoculated milk was distributed into 200 ml bottles. The bottles were incubated in a water bath at 43±1°C and fermented under these 30 conditions until pH 4.5 was reached. The pH was measured continually throughout the fermentation. Subsequently, the bottles were stored at 6°C for 43 days and pH was measured with intervals of 7 days to determine the level of post-acidification.

The acidification profiles of the three commercial starter cultures, F-DVS CH-1, F-DVS YoFlex Mild 2.0, F-DVS YF-L901 and FD-DVS YF-L812, are shown in Figure 5. F-DVS CH-1 showed 35 fast fermentation time reaching pH 4.55 in 4.87 hours. F-DVS YoFlex Mild 2.0 showed intermediate fermentation time reaching pH 4.55 in 5.29 hours. FD-DVS YF-L812 and F-DVS YF-L901 showed slower fermentation reaching pH 4.55 in 6.45 and 5.87 hours, respectively. Post-acidification profiles showed very low levels of post-acidification for FD-DVS YF-L812 and

F-DVS YoFlex Mild 2.0 ( $\Delta\text{pH}=0.12$  and  $\Delta\text{pH}=0.11$  after storage at 6°C for 43 days, respectively), intermediate levels of post-acidification for F-DVS YF-L901 ( $\Delta\text{pH}=0.26$  after storage at 6°C for 43 days and high degree of post-acidification for F-DVS CH-1 ( $\Delta\text{pH}=0.55$  after storage at 6°C for 43 days) (Figure 6).

5

**Example 6:****Effect of the nine *Lb. fermentum* strains on acetaldehyde content when fermented with two different starter cultures**

10 Nine *Lb. fermentum* strains were tested for their ability to lower acetaldehyde content.

Reduced-fat (1.5% w/v) homogenized milk was heat-treated at 90±1°C for 20 min and cooled immediately. Milk was inoculated with one of two commercial starter cultures (F-DVS CH-1 or FD-DVS YF-L812) at 0.02% (v/w), and the inoculated milk was distributed into 200  
15 ml bottles. Nine bottles were inoculated with the *Lb. fermentum* strains in concentrations of 1 x 10<sup>7</sup> CFU/g and one bottle inoculated with each starter culture was used as a reference and only inoculated with the starter culture. All bottles were incubated in a water bath at 43±1°C and fermented at these conditions until pH of 4.55±0.1 was reached. After fermentation, the bottles were vigorously shaken to break the coagulum and cooled on ice. The bottles were  
20 stored at 7±1°C for 14 days.

The tested *Lb. fermentum* strains were: *Lb. fermentum* CHCC12798, *Lb. fermentum* CHCC12797, *Lb. fermentum* CHCC14591, *Lb. fermentum* CHCC14588, *Lb. fermentum* CHCC15844, *Lb. fermentum* CHCC15865, *Lb. fermentum* CHCC15847, *Lb. fermentum* CHCC15926, and *Lb. fermentum* CHCC2008.

On day 14 samples were analyzed for acetaldehyde by static head space gas chromatography (HSGC), a sensitive method for analyzing volatiles in complex matrices. The setup consisted of a Static Head Space sampler connected to Gas Chromatograph with Flame Ionization  
30 Detector (FID). For that purpose the following equipment was used:

HS-autosampler: HS40XI, TurboMatrix 110, Perkin Elmer.

HS-software: HSControl v.2.00, Perkin Elmer.

GC: Autosystem XL, Perkin Elmer.

35 GC-software: Turbochrom navigator, Perkin Elmer.

Column: HP-FFAP 25 m x 0.20 mm x 0.33 µm, Agilent Technologies

Standards of known concentration were used to determine response factors (calibration), controls were used to control that the used response factors were stable within an analytical series as well as in-between series and over time (months). Concentration of volatiles (ppm) in samples and controls was determined using response factors coming from standards.

5 Samples were prepared by adding 200  $\mu$ l of 4N H<sub>2</sub>SO<sub>4</sub> to 1 g yoghurt sample and immediately analyzed by HSGC.

The results are illustrated in Figure 7 and show that each of the strains *Lb. fermentum* CHCC12798, *Lb. fermentum* CHCC12797, *Lb. fermentum* CHCC14591, *Lb. fermentum* CHCC14588, *Lb. fermentum* CHCC15844, *Lb. fermentum* CHCC15865, *Lb. fermentum* CHCC15847, *Lb. fermentum* CHCC15926, and *Lb. fermentum* CHCC2008 has the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product.

15

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#### DEPOSITS and EXPERT SOLUTION

30

The applicant requests that a sample of the deposited micro-organisms stated below may only be made available to an expert, until the date on which the patent is granted.

The *Lactobacillus fermentum* strain CHCC12798 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32084.

The *Lactobacillus fermentum* strain CHCC12797 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32085.

5

The *Lactobacillus fermentum* strain CHCC14591 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32086.

10

The *Lactobacillus fermentum* strain CHCC14588 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32087.

15

The *Lactobacillus fermentum* strain CHCC15844 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32088.

20

The *Lactobacillus fermentum* strain CHCC15865 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32089.

25

The *Lactobacillus fermentum* strain CHCC15847 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32090.

30

The *Lactobacillus fermentum* strain CHCC15848 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32091.

35

The *Lactobacillus fermentum* strain CHCC15926 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und

Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-22 under the accession No.: 32096.

5 The *Lactobacillus fermentum* strain CHCC2008 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2009-05-19 under the accession No.: 22584.

10 The *Lactobacillus rhamnosus* strain CHCC15860 was deposited at German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSMZ), Inhoffenstr. 7B, D-38124 Braunschweig deposited on 2015-07-16 under the accession No.: 32092.

15 The deposits were made according to the Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure.

## CLAIMS

1. Bacterium of the species *Lactobacillus fermentum*, wherein the bacterium is characterized in that it increases the pH of a fermented milk product comprising the *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*.  
5
2. Bacterium of the species *Lactobacillus fermentum* according to claim 1, wherein the increase in pH is at least by a value of 0.1.  
10
3. Bacterium of the species *Lactobacillus fermentum* according to claims 1 or 2, wherein the increase in pH is determined after storing a product fermented with a starter culture and the *Lactobacillus fermentum* in a concentration of at least  $10^7$  cfu/g over  
15 21 days at 25 °C.
4. Bacterium of the species *Lactobacillus fermentum* characterized in that a fermented milk product comprising the *Lactobacillus fermentum* maintains a pH above 4.0 when stored for at least 14 days at 25 °C, wherein the fermented milk product is obtained  
20 by a method comprising incubating a milk with the *Lactobacillus fermentum* in a concentration of at least  $10^7$  CFU/g and with a starter culture, fermenting until a pH of 4.6 is reached, shaking the fermented product and cooling.
5. Bacterium of the species *Lactobacillus fermentum* according to any one of claims 1 to  
25 4, wherein the starter culture used for the preparation of the fermented milk product comprises LAB which are able to decrease the pH of a milk product during fermentation to a value of pH 4.6 in 10 hours or less.
6. Bacterium of the species *Lactobacillus fermentum* according to any one of claims 1 to  
30 5, wherein the starter culture comprises a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.
7. Bacterium of the species *Lactobacillus fermentum* according to any one of claims 1 to  
35 6, wherein the bacterium has the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50%.

8. Bacterium of the species *Lactobacillus fermentum* according to any one of claims 1 to 7, wherein the bacterium has the ability to reduce the concentration of acetaldehyde produced by a starter culture during fermentation in a fermented milk product by at least 50%, at least 75%, at least 95% or at least 98% wherein the concentration of acetaldehyde is determined in an assay comprising:

- (1) preparing a fermented milk product by:
  - (a) inoculating a milk with the *Lactobacillus fermentum* in a concentration of at least  $10^7$  CFU/g and with a starter culture,
  - (b) fermenting until a pH of 4.6 is reached, and;
- (2) storing the fermented milk product at  $7\pm1^\circ\text{C}$  for 14 days;
- (3) adding 200  $\mu\text{l}$  of 4N  $\text{H}_2\text{SO}_4$  to 1 g of the fermented milk product and determining the concentration of acetaldehyde by static head space gas chromatography.

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9. Bacterium of the species *Lactobacillus fermentum* according to any one of claims 1 to 8, wherein the bacterium is selected from the group consisting of:

- (a) the *Lactobacillus fermentum* strain deposited as DSM32084;
- (b) the *Lactobacillus fermentum* strain deposited as DSM32085;
- (c) the *Lactobacillus fermentum* strain deposited as DSM32086;
- (d) the *Lactobacillus fermentum* strain deposited as DSM32087;
- (e) the *Lactobacillus fermentum* strain deposited as DSM32088;
- (f) the *Lactobacillus fermentum* strain deposited as DSM32089;
- (g) the *Lactobacillus fermentum* strain deposited as DSM32090;
- (h) the *Lactobacillus fermentum* strain deposited as DSM32091;
- (i) the *Lactobacillus fermentum* strain deposited as DSM32096;
- (j) the *Lactobacillus fermentum* strain deposited as DSM22584; or
- (k) a mutant strain obtainable from one the deposited bacteria according to (a) to (j), wherein the mutant is characterized in that it increases the pH of a fermented milk product comprising the mutant *Lactobacillus fermentum* during storage after fermentation in comparison to a milk product fermented with the same starter culture not containing the *Lactobacillus fermentum*.

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35 10. Composition comprising at least one *Lactobacillus fermentum* strain according to any one of claims 1 to 9.

11. The composition according to claim 10, wherein the composition further comprises at least one further bacterium selected from:

5 (a) *Lactobacillus rhamnosus* bacterium of strain CHCC15860 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM32092;

10 (b) *Lactobacillus rhamnosus* bacterium of strain CHCC5366 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM23035;

15 (c) *Lactobacillus rhamnosus* bacterium of strain CHCC12697 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24616;

(d) *Lactobacillus paracasei* bacterium of strain CHCC12777 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24651; and

20 (e) *Lactobacillus paracasei* bacterium of strain CHCC14676 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM25612.

12. Composition according to claim 10 or 11, further comprising at least one cryoprotective compound.

25 13. Composition according to any one of claims 10 to 12, wherein the composition is a solid frozen or freeze dried starter culture comprising lactic acid bacteria in a concentration of at least  $10^9$  colony forming units per g of frozen material or in a concentration of at least  $10^{10}$  colony forming units per g of frozen material or in a concentration of at least  $10^{11}$  colony forming units per g of frozen material.

30 14. Method of producing a fermented milk product comprising adding a *Lactobacillus fermentum* bacterium according to any one of claims 1 to 9 or the composition according to any one of claims 11 to 13 to milk or to a milk product and fermenting the mixture at a temperature between about 22°C and about 43°C until a pH of 4.6 or less than 4.6 is reached.

35 15. The method according to claim 14, comprising adding a *Lactobacillus fermentum* bacterium according to any one of claims 1 to 5 or the composition according to any one of claims 6 to 9 to milk or to a milk product and fermenting the mixture

- (a) such that the concentration of the *Lactobacillus fermentum* bacteria of claim 1 is at least  $1\times 10^6$  cfu/g or at least  $1\times 10^7$  cfu/g at the termination of fermentation in the fermented milk product; and/or
- (b) such that the concentration of the *Lactobacillus fermentum* bacteria of claim 1 is at least  $1\times 10^5$  cfu/cm<sup>2</sup> on the surface of the fermented milk product.

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16. The method according to claim 14 or 15, wherein the fermented product is stored at a temperature above 7°C, preferably at a temperature between 7°C and 25°C.

10 17. The method according to one of claims 14 to 16, wherein the fermented product is stored for a period of at least 14 days and wherein the pH of the fermented milk product is maintained above pH 4.0.

15 18. Method of producing a food, feed or pharmaceutical product comprising a method of producing a fermented milk product according to any one of claims 14 to 17.

19. Food, feed or pharmaceutical product obtainable by a method of claim 18.

20. Food, feed or pharmaceutical product comprising a bacterium of the species *Lactobacillus fermentum* selected according to one of claims 1 to 9 and one or more of:

25

- (a) least one further bacterium selected from one or more of the following genera *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp. and *Enterococcus* spp.;
- (b) *Lactobacillus rhamnosus* bacterium of strain CHCC15860 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM32092;
- (c) *Lactobacillus rhamnosus* bacterium of strain CHCC5366 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM23035;
- (d) *Lactobacillus rhamnosus* bacterium of strain CHCC12697 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24616;
- (e) *Lactobacillus paracasei* bacterium of strain CHCC12777 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM24651; and

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(f) *Lactobacillus paracasei* bacterium of strain CHCC14676 as deposited with the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession No. DSM25612.

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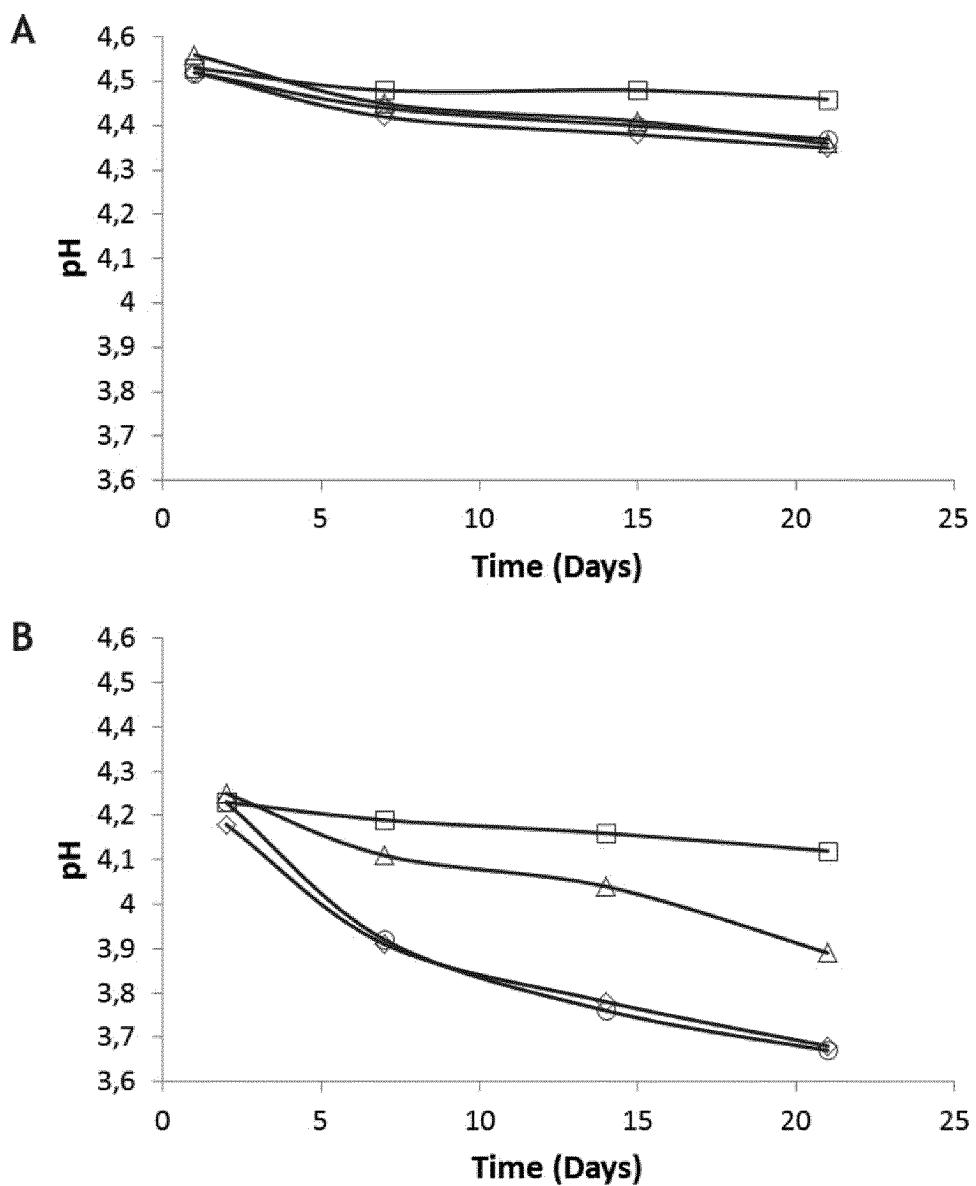


Figure 1

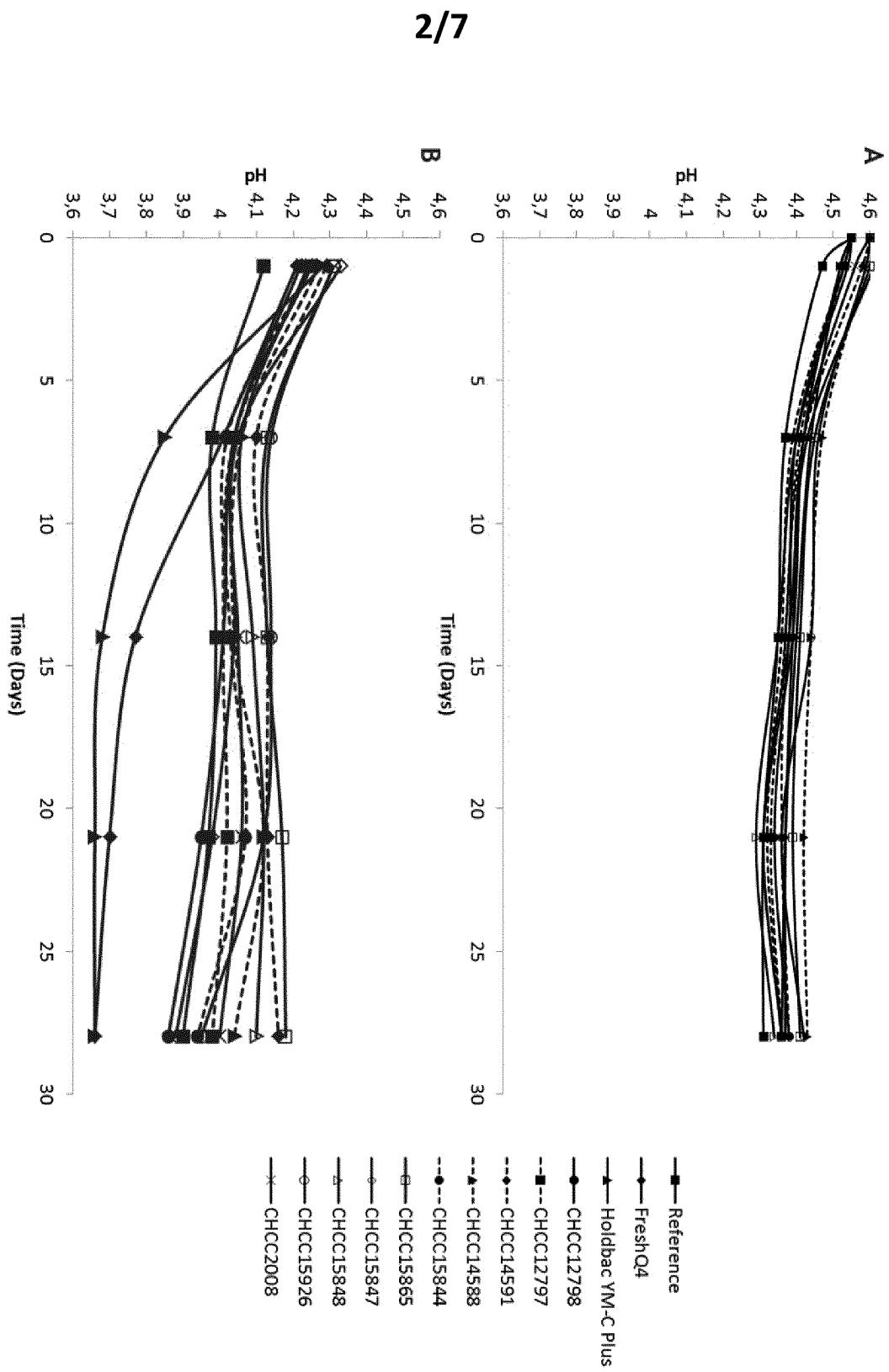


Figure 2

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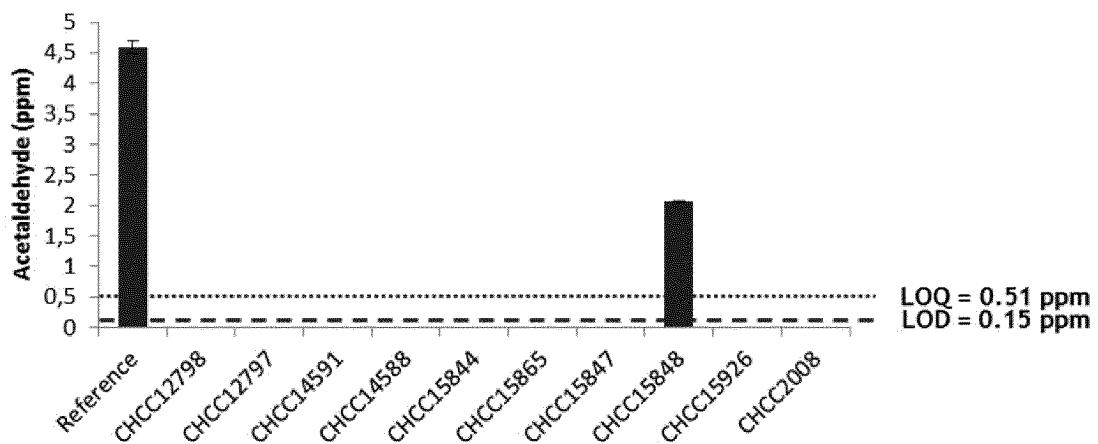
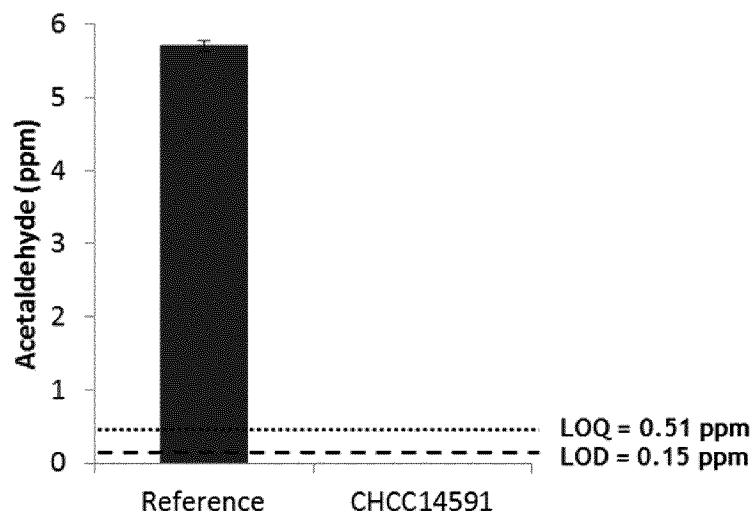


Figure 3

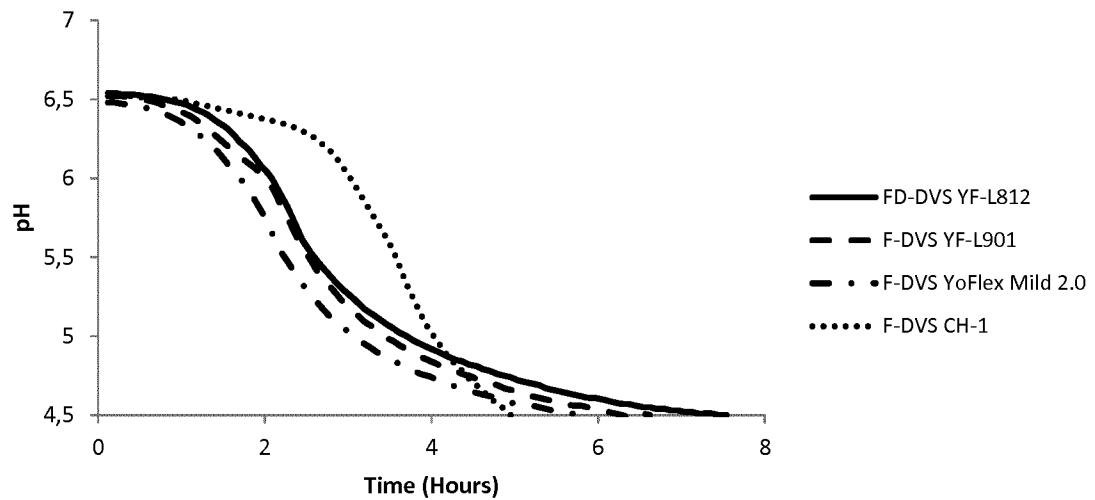
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Figure 4



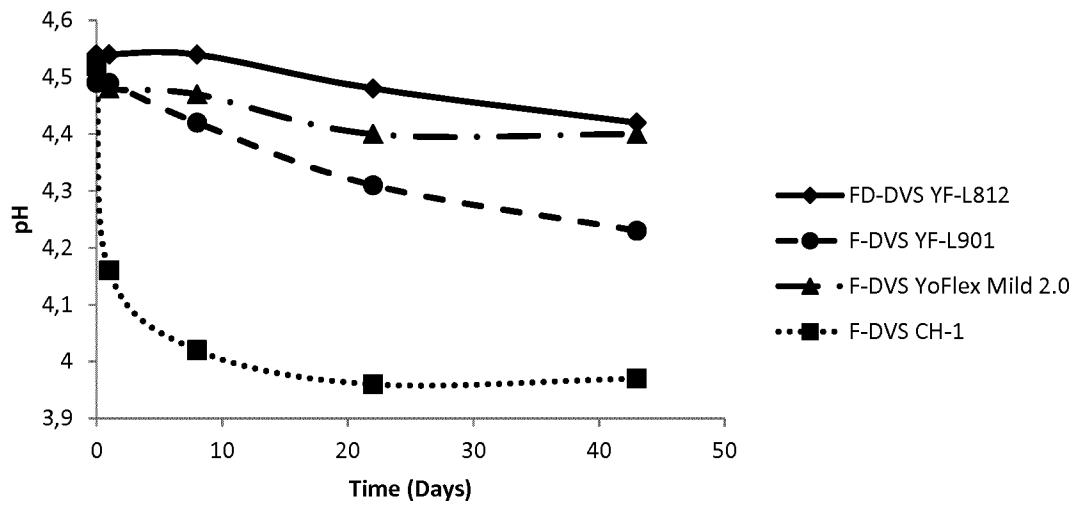
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Figure 5



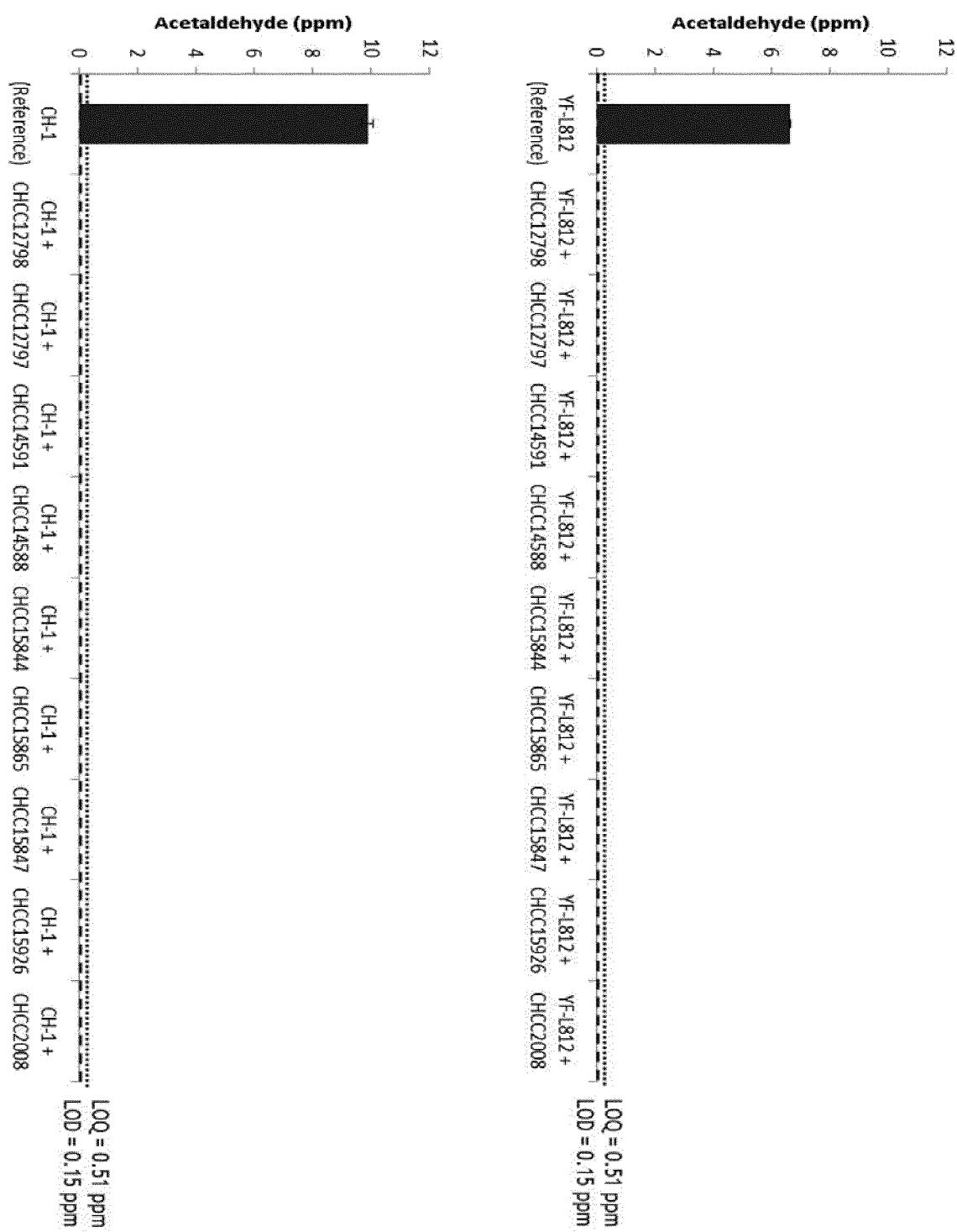
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Figure 6



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Figure 7



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2016/070398

**A. CLASSIFICATION OF SUBJECT MATTER**  
INV. A23C9/123 C12R1/225  
ADD.

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
A23C C12R

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/000879 A2 (CHR HANSEN AS [DK]; FOLKENBERG DITTE MARIE [DK]; POULSEN LONE [DK]) 6 January 2011 (2011-01-06) claim 21; examples 1, 2; tables 1, 2 -----	1-10, 12-20
Y	BILGIN B ET AL: "Assessment of novel human origin Lactobacillus isolates for the manufacture of probiotic yoghurt-like product", MILCHWISSENSCHAFT, VV GMBH VOLKSWIRTSCHAFTLICHER VERLAG. MUNCHEN, DE, vol. 65, no. 1, 1 January 2010 (2010-01-01), pages 65-69, XP008133925, ISSN: 0026-3788 page 66, paragraph 2.2; table 1 page 67, paragraphs 3.1, 3.2, 3.4; figures 1-3, 6 -----	11
X		1-8,10, 14,15, 17-20

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2016	16/11/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Smeets, Dieter

## INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/070398

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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