



Method for preparing a probiotic powder using a two in one whey containing nutrient medium

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(54) Title: METHOD FOR PREPARING A PROBIOTIC POWDER USING A TWO-IN-ONE WHEY-CONTAINING NUTRIENT MEDIUM

(57) Abstract: The present invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising: a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium; b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.



**METHOD FOR PREPARING A PROBIOTIC POWDER USING A TWO-IN-ONE
WHEY-CONTAINING NUTRIENT MEDIUM**

Field of the invention

5 The present invention relates to the field of manufacturing probiotic-containing compositions.

Background of the invention

10 According to the definition of FAO/WHO, probiotics consist in viable microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO, 2001). Nowadays, food products that incorporate probiotics are usually categorized as functional foods due to the increasing evidences of the health benefits reported in both *in vitro* and *in vivo* experiments (Kamada et al., 2013; Pessione, 2014; Rodgers, 2008). For instance, *Propionibacterium freudenreichii* probiotic potential
15 includes immunomodulation by *P. freudenreichii* strain ITG 20 via key surface proteins (Cousin et al., 2012; Cousin et al., 2010; Foligné et al., 2010; Foligné, et al., 2013). Similarly, *Lactobacillus casei* BL23 is an anti-inflammatory strain, also known to attenuate colitis.

20 As a consequence of the increasing demand of these probiotic foods in the recent decades, the current scientific and technical bottleneck addresses the preservation of the probiotic viability during production process, long-term storage and digestion.

25 Drying of bacteria has been recognized as an efficient approach to stabilize the bacterial cells and prolong the shelf life of biological products. Besides, the drying matrix may encapsulate the probiotic cells after drying, which has been proven to exert a protective effect against the adverse conditions during storage and delivery in the digestive tracts (Cook et al., 2012; Paéz et al., 2012; Semyonov et al., 2010; Yonekura et al., 2014).

30 Up to date, freeze drying is still the most frequently used technique for drying of bacteria. Although the conditions (cold temperature, sublimation of water) used in this process are generally beneficial for maintaining the bacteria viability, its relatively high cost and low energy efficiency have been considered as a stumbling block when facing the increasing commercial demand of probiotic products (Holzapfel, 2014; Lievense & van't Riet, 1993; Peighambardoust et al., 2011).

Alternatively to freeze drying, spray drying has been recognized as an efficient technique to produce large amount of dried probiotics. The advantages of spray drying mainly consist in the relatively low production cost, approximately 10 times cheaper than freeze drying, the ability in large-scale production and its mature application in dairy and pharmaceutical industries (Santivarangkna et al., 2007; Schuck et al., 2013). However, the challenge of utilizing spray drying to produce probiotic powders is primarily related to the existence of high temperature during process, which may cause irreversible damages to bacterial cells and may subsequently negatively impact their viability (Ananta et al., 2005; Fu & Etzel, 1995; Lievense & van't Riet, 1994).

Extensive investigations have been carried out in order to improve the remaining viability of probiotic bacteria after spray drying and during following storage. The strategies include process optimization, application of protectants and enhancement of cellular resistance (Desmond et al., 2001; Fu & Chen, 2011; Liu et al., 2015; Peighambardoust et al., 2011). However, these strategies usually and merely focus on the drying step, rarely considering the overall process from growth to drying of bacteria. Therefore, standard laboratory media were used to grow bacteria in most studies (Barbosa et al., 2015; Golowczyc et al., 2010; Lavari et al., 2014; Lian et al., 2002; Perdana et al., 2013; Sunny-Roberts & Knorr, 2009).

For example, it was shown that MRS (de Man et al., 1960) and Yeast Extract Sodium Lactate (YEL) (Malik et al., 1968) broths represent generally standard growth medium of *Lactobacillus sp.* and *Propionibacterium sp.* respectively, corresponding to a total solid content (TS) of about 5 wt%. However, using standard growth conditions has several adverse consequences when using these media as raw materials in industrial spray drying: in particular, low powder flow rate, high evaporation rate and drying temperature needed, and occurrence of undesired caking or sticking in the relatively fine powders (Stoklosa et al., 2012; Wu et al., 2014). Conversely, an inhibition of the bacterial growth is expected when increasing the medium total solid (TS) content, because of the higher osmotic stress (Chirife et al., 1983; Vasseur et al., 1999; Walter et al., 1987).

When the probiotics cells have been grown in these standard nutrient media, the subsequent operations required before the accessing to the drying unit include rinsing, harvesting and re-suspending of bacteria. There are several disadvantages in this conventional process of spray drying of bacteria. From the practical point of view, the

standard laboratory media used for growth of bacteria, e.g. the most frequently MRS, are normally expensive and non-food grade (De Man et al., 1960). In addition to the waste of material, the removal of these media may lead to the loss of bacteria viability and increase the risk of contamination during the intermediate operations such as rinsing, centrifugation and re-suspension. The possible residual components on the bacteria pellets from the media may also cause interference to the subsequent operation and further application.

Furthermore, documents such as FR 2 802 212, EP 0 818 529 and WO 01/36590 have disclosed the benefits of co-pulverization of probiotics obtained from standard growth together with protective agents, such as, sweet whey concentrate, concentrated milk, lactose, saccharose, trehalose, galactose, starch, sorbitol, casein, beta-lactoglobulin, alpha-lactalbumin, soy, serum albumin, glutenin, prolamin, lysine, cysteine, glycine, vitamins, in order to improve their survival after spray drying.

In the same line, Jantzen et al. (2013), discloses a spray drying method of *L. reuteri* comprised in a watery 20% whey solution complemented with 0.5% (w/v) of yeast extract.

Finally, RU 2010 140092 and CS 9 100 190 disclose a spray drying method of probiotics in the presence of 20-25% (w/v) whey.

Therefore, there is a need to provide new means to improve bacterial survival after a spray drying process.

There is also a need for simplifying the spray drying process.

Summary of the invention

In one aspect, the invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from above 25% by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium;

b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

In another aspect, the invention also relates to a probiotic powder comprising at least one probiotic bacterium obtained by a method according to the invention.

In another aspect, the invention also relates to the probiotic powder according to the invention for use for improving health of a human or an animal body.

In a still another aspect, the invention relates to the use of a whey-containing nutrient medium having a total solid content ranging from 15 % by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, for culturing at least one probiotic bacterium, for the preparation of a probiotic powder comprising the said at least one probiotic bacterium.

Another aspect of the invention relates to the use of a whey-containing nutrient medium having a total solid content ranging from 15 % by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, said whey from the whey-containing nutrient medium being the exclusive nitrogen source, for culturing at least one probiotic bacterium, for the preparation of a probiotic powder comprising the said at least one probiotic bacterium.

In another aspect, the invention relates to the use of a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, for culturing at least one probiotic bacterium, for the preparation of a probiotic powder comprising the said at least one probiotic bacterium.

Legends of the figures

Figure 1. Plots illustrating the final bacteria populations (left Y axis) and the dependency (right Y axis; diamonds) of bacteria growth on casein peptone of **(A)** *L. casei* and **(B)** *P. freudenreichii* in the sweet whey nutrient media with different total solid content (TS) and with (dark gray bars) or without (light gray bars) supplement of casein peptone (in comparison to the standard media, i.e., MRS broth for *L. casei* and YEL broth for *P. freudenreichii*). Mean \pm SEM, n = 6. Different letters: significantly different between the bacteria population ($p < 0.05$); Different number of *: significantly different between the dependency ($p < 0.05$).

Figure 2. Plots illustrating the remaining bacteria populations (left Y axis; gray bars) and survival (right Y axis; diamonds) of **(A)** *L. casei* and **(B)** *P. freudenreichii* in the sweet whey media with different TS after spray drying; **(C)** Plot illustrating the survival of bacteria growing and spray drying in the 5% media (1), growing in 5%

medium but being added to 30% before drying (2), growing and spray drying in 30% media (3). Mean \pm SEM, n = 3. Different letters: significantly different between the bacteria population ($p < 0.05$); Different number of *: significantly different between the survival ($p < 0.05$).

Figure 3. Plots illustrating the volume of particle size of the spray dried powders of **(A)** *L. casei* and **(B)** *P. freudenreichii* from the sweet whey media with different TS, 5% (curve 1), 10% (curve 2), 20% (curve 3), 30% (curve 4) and 40% (curve 5). The curves were obtained from the average value of duplicate experiments. The size range was measured from 0.1 to 2000 μm ; the values of volume at the ranges from 0.1 to 1 μm and from 200 to 2000 μm were all 0.

Figure 4. Plots illustrating the change of bacteria populations in the spray dried powders of **(A)** *L. casei* and **(B)** *P. freudenreichii* from the sweet whey media with different TS, 5% (diamonds), 10% (squares), 20% (triangles), 30% (large circles) and 40% (small circles), during storage at 4 °C for 120 days. Mean \pm SEM, n = 3. **(C)** Plots illustrating the final log reduction of two probiotics strains *L. casei* (circles) and *P. freudenreichii* (squares) when storage for 120 days. Different number of *: significantly different between the survival ($p < 0.05$).

Detailed description of the invention

The inventors have surprisingly found that it is possible to use a concentrated whey-containing nutrient medium to grow probiotic bacteria in order to achieve a two-step spray drying process comprising (i) growing the probiotics and (ii) drying said probiotics culture. The inventors have overcome the prejudices in the art acknowledging (1) the putative inhibition of probiotics growth when using a nutrient medium with high total solid content and (2) the need for artificially increasing the total solid content of the culture obtained with the use of “standard” growth conditions, just prior spray drying or freeze drying.

Furthermore, the inventors show for the first time that probiotic bacteria may be cultured in a whey-containing nutrient medium having a total solid content of whey of above 25 wt% and up to 35 wt%. Indeed, the skilled person in the art had the prejudice that a total solid content of whey up to 25 wt% could not be used to grow probiotics.

In addition, the inventors found that the whey may constitute the exclusive nitrogen source in the whey-containing nutrient medium, as to provide for the growth of the probiotic bacteria.

5 **Method**

In one aspect, the invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing
10 nutrient medium having a total solid content ranging from 15% by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium;

b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

Within the scope of the invention, a “probiotic powder” is intended to refer to a
15 particulate product comprising live microorganisms that are intended to impart health benefits when consumed by a living animal or human body in adequate amounts.

In particular, health benefits may encompass the prevention or the treatment of e.g. digestive disorders, such as diarrhea, lactose intolerance, colitis, liver disease, irritable
20 bowel syndrome and inflammatory bowel disease; allergic disorders, such as atopic dermatitis and allergic rhinitis; oral health problems. Some other benefits of probiotics also include immune stimulation, enhancement of bowel mobility, reduction of inflammatory reactions and even cancer apparition.

Within the scope of the invention, a “biomass composition” is intended to mean living probiotic microorganisms and/or any biological material derived from said
25 living, or recently living probiotic microorganisms. A biomass composition according to the invention includes living probiotics together with unconsumed compounds from the nutrient medium and by-products of the probiotics metabolism.

Within the scope of the invention, the terms “culture” and “growth” are intended to be equivalent and may be therefore substituted to one another, and further
30 refer to the step of multiplying viable probiotics to achieve a desired biomass composition.

Within the scope of the invention, a “nutrient medium” is meaning a nutrient composition comprising at least (i) one or more pH buffering system(s); (ii) inorganic

salts; (iii) trace elements; (iv) free amino acids; (v) vitamins; (vi) one or more carbon/energy source(s) and suitable for cultivating at least one probiotic bacterium under appropriate growth conditions.

5 Within the scope of the invention, a “solid content” of any given compound in a mixture refers to the solid weight of said component as compared to the total solid (TS) weight of said mixture. In practice, the solid content of any given compound is expression in percent (%) by weight, as compared to the total weight of the said composition.

10 Within the scope of the invention, the expressions “a nutrient medium having a total solid content of ‘X’ wt%”, “a nutrient medium having a total solid content of ‘X’ % by weight” and “a nutrient medium having a total solid content of ‘X’ wt%, as compared to (or based on) the total weight of the said nutrient medium” are intended to be equivalent and may be substituted to one another.

15 In practice, the total solid (TS) content of an aqueous liquid substance may be measured by evaporating the liquid in an oven at a temperature of at least 100°C for a sufficient time to collect a dry powder, which is subsequently weighted. The determined dried weight is then compared to the total weight of the liquid substance before evaporation.

20 Within the scope of the invention, “whey” is intended to mean, according to its conventional meaning in the art, the by-product substance originated from cheese production.

- Probiotic bacteria

25 Within the scope of the invention, the terms and expressions “probiotic”, “probiotic bacterium” and “probiotic microorganism” are intended to be equivalent and may be therefore substituted to one another.

 In some embodiments, said probiotic bacterium is selected in a group comprising *Bifidobacterium sp.*, *Lactobacillus sp.*, *Lactococcus sp.*, *Propionibacterium sp.*, *Streptococcus sp.* and a mixture thereof.

30 In some embodiments, said probiotic bacterium is selected in a group comprising *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*; *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri*, *L.*

paracasei, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. rhamnosus*, *Lactococcus lactis*, *Propionibacterium freudenreichii*, *Streptococcus thermophilus* and a mixture thereof.

In some embodiments, said probiotic bacterium is selected in a group comprising *Lactobacillus acidophilus*, *L. amylovorus*, *L. brevis*, *L. casei*, *L. rhamnosus*, *L. crispatus*, *L. delbrueckii subsp. bulgaricus*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. lactis*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. gallinarum* and a mixture thereof.

In some embodiments, said probiotic bacterium is selected in a group comprising *Bifidobacterium adolescentis*, *B. animalis*, *B. breve*, *B. bifidum*, *B. infantis*, *B. lactis*, *B. longum* and a mixture thereof.

In some embodiments, said probiotic bacterium is selected in a group comprising *Bacillus cereus*, *Clostridium botyricum*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. lactis*, *Leuconostoc mesenteroides subsp. dextranicum*, *Pediococcus acidilactici*, *Propionibacterium freudenreichii*, *Saccharomyces boulardii*, *Streptococcus salivarius subsp. thermophiles*, *Sporolactobacillus inulinus* and a mixture thereof.

In some preferred embodiments, said probiotic bacterium is selected in a group comprising *L. casei* and *P. freudenreichii*.

In some embodiments, the said probiotic bacterium is a thermoresistant probiotic bacterium.

Within the scope of the invention, a thermoresistant probiotic bacterium is intended to refer to a probiotic bacterium that can sustain temperatures up to 60°C, preferably up to 55°C, without showing impaired survival.

In practice, a thermoresistant probiotic bacterium suitable for implementing the invention may be used in method for preparing cheese, in particular method comprising a step in which the thermoresistant probiotic bacterium is brought to temperature comprised from 45°C to 60°C, preferably from 50°C to 55°C.

In some embodiments, a thermoresistant probiotic bacterium suitable for implementing the invention may be used in method for yogurt, in particular method comprising a step in which the thermoresistant probiotic bacterium is brought to temperature comprised from 38°C to 47°C, preferably from 40°C to 45°C.

- Growth conditions

The inventors have shown that spray drying of a biomass comprising probiotics may be performed without the need of concentrating said biomass prior to achieving spray drying, as disclosed abundantly in the literature.

5 Therefore, the inventors disclose hereby a simple process to grow and dry the probiotics in sweet whey, which was chosen a two-in-one nutrient medium, rendering the steps such as rinsing, centrifugation and re-suspension obsolete.

Within the scope of the invention, a “two-in-one nutrient medium” is intended to mean a nutrient medium that is suitable to perform the step of growing the probiotic
10 bacteria to obtain a probiotic biomass and further adapted to perform spray drying of said biomass, without the need of removing said nutrient medium from said probiotic bacteria after growth.

Within the scope of the invention, a total solid content ranging from 15% to 35% encompasses a total solid content of 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%,
15 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33% and 34%.

In some embodiments, the whey-containing nutrient medium has a total solid content ranging from 20% by weight to 30% by weight, based on the total weight of the said nutrient medium.

In some other embodiments, the whey-containing nutrient medium has a total
20 solid content ranging from above 25% by weight to up to 35% by weight, based on the total weight of the said nutrient medium.

Within the scope of the invention, a total solid content ranging from above 25% to up to 35% encompasses a total solid content of 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%,
25 33.0%, 33.5%, 34.0%, 34.5% and 35.0%. As shown in the example section here below, a biomass composition, resulting from a culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from 15 wt% to 35 wt%, preferably from above 25% to up to 35 wt%, presents a higher viability (as expressed in CFU.mL⁻¹) and a higher survival after spray drying, as compared to a biomass
30 composition resulting from a culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content below 15 wt%, preferably strictly below 26%, or above 35 wt%.

As also shown in the example section here below, a biomass composition, resulting from a culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from 15 wt% to 35 wt%, presents a higher survival after spray drying, as compared to a biomass composition resulting from a culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content below 10 wt%, but which total solid content was adjusted to 15 wt% to 35 wt%, just prior to spray drying.

Without wishing to be bound by any particular theory, the inventors believe that the probiotic biomass obtained in whey-containing nutrient media with high total solid content is tolerant due to the cellular response, e.g., accumulation of intracellular compatible solutes triggered by the high osmolality during growth.

In some embodiments, the whey is sweet whey, which is a by-product of rennet-coagulated cheese. Sweet whey generally has a pH strictly above 5.1.

In some other embodiments, the whey is acid whey, which is a by-product obtained during the making of acid types of dairy products, such as cottage cheese or strained yogurts. Acid whey generally has a pH equal or inferior to 5.1.

In practice, sweet whey may originate from commercially available powder, such as e.g. Lactalis ingredients (France).

In some embodiments, whey-containing nutrient media may be prepared by pouring whey powders into any suitable liquid, such as deionized water, PBS buffer, or standard nutrient medium, such as e.g. MRS (Man Rogosa Sharpe) broth, M17 broth or YEL broth.

In certain embodiments, a whey-containing nutrient medium according to the invention may be prepared by pouring a whey powder into deionized water.

M17 broth is preferably used for the growth of *Lactococcus sp.* MRS broth or vegetable MRS broth is preferably used for the growth of *Lactobacillus sp.* YEL broth is preferably used for the growth of *Propionibacterium sp.*

Indeed, any culture medium known from the one skilled in the art may be used for growing the bacteria of interest, which include culture media based on milk, culture media based on milk permeate, culture media based on molasses or also culture media corn steep liquor.

In some embodiments, the whey-containing nutrient medium may further comprise one or more protective agent(s), known to have a beneficial effect on the survival of the probiotics after spray drying or freeze drying.

5 In some embodiments, said protective agent(s) may be chosen in a group comprising trehalose, saccharose, galactose, starch, sucrose, maltose, lactose, glucose, fructose, sorbitol, dextran, maltodextrin, skim milk (optionally under the form of a skim milk powder), betaine, galactomannane, carraghenane, pectin, casein, beta-lactoglobulin, alpha-lactalbumin, serum albumin, albumin, globulin, glutelin, prolamin, protamine, lysine, cysteine, glycine, glycerol, acacia gum (also termed "Arabic gum") and a mixture
10 thereof.

In some embodiments, casein may be provided in the form of casein peptone as a preferred nitrogen source.

In some certain embodiments, said nutrient medium may further comprise casein peptone.

15 In some embodiments, the casein peptone may represent from 0.1% to 1% (w/w) of the weight of the nutrient medium.

Surprisingly, the inventors have noticed that the whey comprised in the whey-containing nutrient medium may be the exclusive nitrogen source. As shown in the example section below, above a total solid content of whey of the whey containing
20 medium of 20 wt%, as the total solid content of whey of the whey containing medium increases, the dependency of growth towards external additional nitrogen source decreases.

In other words, the whey-containing nutrient medium may be free or substantially free of an external additional nitrogen source.

25 Within the scope of the invention, the expression "substantially free of an additional nitrogen source" is intended to mean a whey-containing nutrient medium comprising strictly less than 2.5 wt% of an additional nitrogen source, preferably less than 1 wt%, more preferably less than 0.5 wt%, based on the total weight of the said nutrient medium.

30 In practice, an external additional nitrogen source may be selected in the group comprising yeast extract, beef extract, peptone, casein peptone and the like.

In some embodiments, the whey-containing nutrient medium is free or substantially free of yeast extract, beef extract, peptone, casein peptone and the like.

One aspect of the invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

- 5 a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from 15 % by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, said whey from the whey-containing nutrient medium being the exclusive nitrogen source;
- 10 b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

Another aspect of the invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

- 15 a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium;
- 20 b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

In some embodiments, the whey-containing nutrient medium has a total solid content ranging from 26 % by weight to 33 % by weight, based on the total weight of the said whey-containing nutrient medium.

25 Another aspect of the invention relates to a method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

- 30 a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, said whey from the whey-containing nutrient medium being the exclusive nitrogen source;

b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

In practice, a pre-culture of at least one probiotic bacterium may be performed in an adequate nutrient medium, with or without whey, before inoculation in the final culture, intended to be spray dried. Such nutrient medium may be as already described above, e.g. M17 broth, MRS broth or YEL broth.

In some embodiments, the nutrient medium is free of yeast extract and/or yeast biomass.

Culture conditions with respect to the temperature, time and agitation may be conform to the culture conditions from the state of the art, depending of the type of probiotic.

In some embodiments, the temperature of the probiotic culture is ranging from about 20°C to about 40°C, preferably ranging from about 30°C to about 37°C.

In some embodiments, a temperature of the probiotic culture ranging from about 20°C to about 40°C includes a temperature of about 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C and 39°C.

In some embodiments, the time length of the probiotic culture is ranging from about 24h to about 150h, preferably from about 48h to about 120h.

In some embodiments, a time length of the probiotic culture ranging from about 24h to about 150h includes a time length of about 24h, 30h, 36h, 42h, 48h, 54h, 60h, 66h, 72h, 78h, 84h, 90h, 96h, 102h, 108h, 114h, 120h, 126h, 132h, 138h, 144h, 150h.

- Biomass composition

In contrast to previously known methods for preparing probiotic-containing powders wherein the biomass composition comprising probiotics that are undergoing spray drying, the method described herein does not comprise a step wherein the final total solid (TS) content is adjusted just prior to spray drying.

According to the method described herein, the final total solid (TS) content may be adjusted during the preparation of the whey-containing nutrient medium, i.e. prior to starting the culture of the probiotic bacterium itself.

As mentioned above, the probiotic biomass composition to be spray dried according to the invention does not require any concentration step in order to artificially control the concentration of probiotic cells and/or the total solid content. In particular, the biomass composition to be spray dried according to the invention does not require any sedimentation, or centrifugation step.

In some embodiments, a biomass composition to be spray dried according to the invention may comprise at least 5×10^7 CFU.mL⁻¹ of said at least one probiotic bacterium.

In some embodiments, a biomass composition comprising at least 5×10^7 CFU.mL⁻¹ of said at least one probiotic bacterium includes 6×10^7 CFU.mL⁻¹, 7×10^7 CFU.mL⁻¹, 8×10^7 CFU.mL⁻¹, 9×10^7 CFU.mL⁻¹, 1.0×10^8 CFU.mL⁻¹, 1.2×10^8 CFU.mL⁻¹, 1.4×10^8 CFU.mL⁻¹, 1.6×10^8 CFU.mL⁻¹, 1.8×10^8 CFU.mL⁻¹, 2.0×10^8 CFU.mL⁻¹, 2.2×10^8 CFU.mL⁻¹, 2.4×10^8 CFU.mL⁻¹, 2.6×10^8 CFU.mL⁻¹, 2.8×10^8 CFU.mL⁻¹, 3.0×10^8 CFU.mL⁻¹, 3.5×10^8 CFU.mL⁻¹, 4.0×10^8 CFU.mL⁻¹, 4.5×10^8 CFU.mL⁻¹, 5.0×10^8 CFU.mL⁻¹, 5.5×10^8 CFU.mL⁻¹, 6.0×10^8 CFU.mL⁻¹, 6.5×10^8 CFU.mL⁻¹, 7.0×10^8 CFU.mL⁻¹, 7.5×10^8 CFU.mL⁻¹, 8.0×10^8 CFU.mL⁻¹, 8.5×10^8 CFU.mL⁻¹, 9.0×10^8 CFU.mL⁻¹, 9.5×10^8 CFU.mL⁻¹, 10^9 CFU.mL⁻¹, 2×10^9 CFU.mL⁻¹, 3×10^9 CFU.mL⁻¹, 4×10^9 CFU.mL⁻¹, 5×10^9 CFU.mL⁻¹, 6×10^9 CFU.mL⁻¹, 7×10^9 CFU.mL⁻¹, 8×10^9 CFU.mL⁻¹, 10^{10} CFU.mL⁻¹, 2×10^{10} CFU.mL⁻¹, 3×10^{10} CFU.mL⁻¹, 4×10^{10} CFU.mL⁻¹, 5×10^{10} CFU.mL⁻¹, 6×10^{10} CFU.mL⁻¹, 7×10^{10} CFU.mL⁻¹, 8×10^{10} CFU.mL⁻¹, 9×10^{10} CFU.mL⁻¹ and 10^{11} CFU.mL⁻¹ of said at least one probiotic bacterium.

- *Spray drying*

Spray drying may be performed according to any suitable known method and using any suitable known spray dryer of the art, as long as it comprises at least the following steps:

- a) atomization, namely the transforming the feed into droplets may be performed by any one of the following techniques:
 - pressure nozzle atomization, wherein the spray is achieved by forcing the fluid through an orifice;
 - two-fluid nozzle atomization, wherein the spray is achieved by mixing the feed with a compressed gas;

- centrifugal atomization, wherein the spray is achieved by passing the feed through or across a rotating disk.
- b) drying: a constant rate phase ensures rapid evaporation of the moisture from the surface of the particle and is followed by a falling rate period, during which the drying is controlled by diffusion of water to the surface of the particle.

In some embodiments, prior to the atomization step, the biomass composition may be homogenised, preferably with mild mechanical agitation, for a minimum of 10 min, preferably for a time ranging from 15 min to 30 min.

In some embodiments, upon drying, the powder may be separated from moist gas. This step may be achieved by removing the fine particles with cyclones, bag filters, precipitators or scrubbers.

In some embodiments, upon drying, the powder may undergo cooling and subsequent packaging.

In some preferred embodiments, the spray drying is performed by two-fluid nozzle atomization, preferably by utilizing a spray dryer commercially available from GEA Niro A/S (Denmark), accordingly to the manufacturer's recommendations.

In some embodiments, the biomass composition according to the invention is sprayed into a gas, preferably having an inlet temperature in the range from 100 to 200°C, in a spray dryer, whereas the spray dryer has an outlet temperature of at most 80°C.

In some embodiments, suitable gases include air, nitrogen, argon, helium, carbon dioxide, and mixtures thereof. In some preferred embodiments, the gas comprises air. In some particular embodiments, the gas comprises dehumidified air. Dehumidification may be performed by any suitable dehumidifier, preferably to achieve a humidity of the inlet air ranging from 0.5 g kg⁻¹ to 0.5 g.kg⁻¹ of water in the air, preferably around 1 g.kg⁻¹.

In some embodiments of step b) of the method, the inlet temperature of spray drying is ranging from 120°C to 200°C, preferably ranging from 140°C to 180°C.

In some embodiments of step b) of the method, an inlet temperature of spray drying ranging from 120°C to 200°C includes an inlet temperature of 130°C, 140°C, 150°C, 160°C, 170°C 180°C and 190°C.

In some embodiments of step b) of the method, the outlet temperature of spray drying is ranging from 55°C to 80°C, preferably ranging from 60°C to 75°C.

In some embodiments of step b) of the method, an outlet temperature of spray drying ranging from 55°C to 80°C includes an outlet temperature of 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C and 79°C.

5

- Freeze drying

Freeze drying may be performed according to any suitable known method in the art and using any suitable equipment of the art, as long as it comprises at least a step of sublimation, intended to remove most or all the water contained in the composition comprising the probiotic biomass from step a).

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In practice, the freeze drying step may comprise a freezing step followed by a step of decrease of the pressure.

In some embodiments, the freezing step may be performed at a temperature comprised from -20°C to -196°C, preferably from -50°C to -80°C.

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In some embodiments, the step of decrease of the pressure may be performed as to obtained a final pressure comprised from 1 Pa to 10,000 Pa, preferably from 10 Pa to 1,000 Pa, most preferably from 25 Pa to 100 Pa.

Probiotic powder

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In one aspect, the invention relates to a probiotic powder comprising at least one probiotic bacterium obtained by a method according to the instant invention.

- Particle size distribution

In some embodiments, the probiotic powder may be characterized by the size distribution of the particles contained in said powder.

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In practice, the particle size distribution of powders, in particular “D0.9”, “D0.5” and “D0.1” values, may be determined by well-known methods of the prior art such as sieve analysis, laser light scattering, photo-analysis or optical counting methods.

In some embodiments, laser light scattering is particularly preferred to determine the size distribution of the particles contained in the probiotic powder.

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Within the scope of the invention, by “D0.9 particle size” is meant that the particle size distribution is such that at least 90% of the particles have a particle size diameter of less than the specified value.

Within the scope of the invention, by “D0.5 particle size” is meant that the particle size distribution is such that at least 50% of the particles have a particle size diameter of less than the specified value.

5 Within the scope of the invention, by “D0.1 particle size” is meant that the particle size distribution is such that at least 10% of the particles have a particle size diameter of less than the specified value.

10 Within the scope of the invention, the term “about” before a “specific value” defines a range from “the specific value minus 10% of the specific value” to “the specific value plus 10% of the specific value”. For example, “about 50” defines a range from 45 to 55.

In some embodiments, D0.9 particle size is less than about 100 μm , which includes D0.9 particle sizes less than about 90 μm , 80 μm , 70 μm , 60 μm , 55 μm , 50 μm , 45 μm , 40 μm , 38 μm , 36 μm , 34 μm , 32 μm , 30 μm , 28 μm , 26 μm , 24 μm , 22 μm and 20 μm .

15 In some embodiments, D0.9 particle size is ranging from about 25 μm to about 90 μm , which includes D0.9 particle sizes of about 30 μm , 35 μm , 40 μm , 45 μm , 50 μm , 55 μm , 60 μm , 65 μm , 70 μm , 75 μm , 80 μm and 85 μm .

20 In some embodiments, D0.5 particle size is ranging from about 8 μm to about 50 μm , which includes D0.5 particle sizes of about 9 μm , 10 μm , 11 μm , 12 μm , 13 μm , 14 μm , 15 μm , 16 μm , 17 μm , 18 μm , 19 μm , 20 μm , 21 μm , 22 μm , 23 μm , 24 μm , 25 μm , 26 μm , 27 μm , 28 μm , 29 μm , 30 μm , 31 μm , 32 μm , 33 μm , 34 μm , 35 μm , 36 μm , 37 μm , 38 μm , 39 μm , 40 μm , 41 μm , 42 μm , 43 μm , 44 μm , 45 μm , 46 μm , 47 μm , 48 μm and 49 μm .

25 In some embodiments, D0.1 particle size is less than about 10 μm , which includes D0.1 particle sizes of less than about 9.5 μm , 9 μm , 8.5 μm , 8 μm , 7.5 μm , 7 μm , 6.5 μm , 6 μm , 5.5 μm , 5 μm , 4.5 μm , 4 μm , 3.5 μm , 3 μm , 2.5 μm and 2 μm .

In some embodiments, D0.1 particle size is ranging from about 6 μm to about 9 μm , which includes D0.1 particle sizes of about 6.2 μm , 6.4 μm , 6.6 μm , 6.8 μm , 7.0 μm , 7.2 μm , 7.4 μm , 7.6 μm , 7.8 μm , 8.0 μm , 8.2 μm , 8.4 μm , 8.6 μm and 8.8 μm .

30 In some embodiments, the probiotic powder comprises particles having:

- a D0.9 particle size of less than about 100 μm , and/or
- a D0.5 particle size ranging from about 8 μm to about 50 μm , and/or

- a D0.1 particle size of less than about 10 μm .

In some embodiments, the probiotic powder comprises particles having:

- a D0.9 particle size ranging from about 25 μm to about 90 μm , and/or
- a D0.5 particle size is ranging from about 8 μm to about 50 μm , and/or
- a D0.1 particle size ranging from about 6 μm to about 9 μm .

In some embodiments, the size distribution of the particles is characterized by a ratio $D0.5 (TS\ 15-35\%)/D0.5 (TS \leq 5\%) > 1$, wherein:

- “D0.5(TS 15-35%)” represents a D0.5 particle size measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content ranging from 15 % by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, and

- “D0.5(TS $\leq 5\%$)” represents a D0.5 particle size measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content equal or below 5 % by weight, based on the total weight of the said whey-containing nutrient medium.

In some embodiments, a ratio $D0.5 (TS\ 15-35\%)/D0.5 (TS \leq 5\%) > 1$ includes a ratio equal or above about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.5, 5.0, 5.5 and 6.0.

In some embodiments, the size distribution of the particles of the probiotic powder is characterized by a ratio $D0.5 (TS\ 15-35\%)/D0.5 (TS \leq 5\%) > 1.3$.

In some embodiments, the “Span” parameter is used to characterize the size distribution of powders, and is calculated as follows:

$$\text{Span} = (D0.9 - D0.1) / D0.5$$

wherein D0.1, D0.5 and D0.9 represent the above defined parameters.

In some embodiments, the size distribution of the particles of the probiotic powder is characterized by a Span ranging from 1.2 to 3.0, preferably ranging from 1.4 to 2.8.

In some embodiments, a Span ranging from 1.2 to 3.0 includes 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9.

In some embodiments, the size distribution of the particles is characterized by a ratio $\text{Span} (TS\ 15-35\%)/\text{Span} (TS \leq 5\%) > 1$, wherein:

- “Span(TS 15-35%)” represents a Span value calculated from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content ranging from 15 to 35 wt%, and

- “Span(TS $\leq 5\%$)” represents a Span value calculated from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content equal or below 5 wt%.

In some embodiments, a ratio Span (TS 15-35%)/Span (TS $\leq 5\%$) >1 includes a ratio equal or above about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.5, 5.0, 5.5 and 6.0.

In some embodiments, the size distribution of the particles is characterized by a ratio $D_{0.5} \text{ (TS 15-35\%)} / D_{0.5} \text{ (TS } \leq 5\%) \geq 1.3$ and/or a ratio Span (TS 15-35%)/Span (TS $\leq 5\%) \geq 1.3$.

- Viability

In some embodiments, the probiotic powder may be characterized by a number of viable cells (CFU, colony forming units) per gram (CFU.g⁻¹) of said powder.

In some embodiments, the probiotic powder comprises at least 10⁸ CFU.g⁻¹ of said at least one probiotic bacterium.

In some embodiments, 10⁸ CFU.g⁻¹ of said at least one probiotic bacterium includes 1.2x10⁸ CFU.g⁻¹, 1.4x10⁸ CFU.g⁻¹, 1.6x10⁸ CFU.g⁻¹, 1.8x10⁸ CFU.g⁻¹, 2.0x10⁸ CFU.g⁻¹, 2.2x10⁸ CFU.g⁻¹, 2.4x10⁸ CFU.g⁻¹, 2.6x10⁸ CFU.g⁻¹, 2.8x10⁸ CFU.g⁻¹, 3.0x10⁸ CFU.g⁻¹, 3.5x10⁸ CFU.g⁻¹, 4.0x10⁸ CFU.g⁻¹, 4.5x10⁸ CFU.g⁻¹, 5.0x10⁸ CFU.g⁻¹, 5.5x10⁸ CFU.g⁻¹, 6.0x10⁸ CFU.g⁻¹, 6.5x10⁸ CFU.g⁻¹, 7.0x10⁸ CFU.g⁻¹, 7.5x10⁸ CFU.g⁻¹, 8.0x10⁸ CFU.g⁻¹, 8.5x10⁸ CFU.g⁻¹, 9.0x10⁸ CFU.g⁻¹, 9.5x10⁸ CFU.g⁻¹, 10⁹ CFU.g⁻¹, 2x10⁹ CFU.g⁻¹, 3.10⁹ CFU.g⁻¹, 4x10⁹ CFU.g⁻¹, 5x10⁹ CFU.g⁻¹, 6x10⁹ CFU.g⁻¹, 7x10⁹ CFU.g⁻¹, 8x10⁹ CFU.g⁻¹, 9x10⁹ CFU.g⁻¹, 10¹⁰ CFU.g⁻¹, 2x10¹⁰ CFU.g⁻¹, 3.10¹⁰ CFU.g⁻¹, 4x10¹⁰ CFU.g⁻¹, 5x10¹⁰ CFU.g⁻¹, 6x10¹⁰ CFU.g⁻¹, 7x10¹⁰ CFU.g⁻¹, 8x10¹⁰ CFU.g⁻¹, 9x10¹⁰ CFU.g⁻¹ and 10¹¹ CFU.g⁻¹ of said at least one probiotic bacterium.

In practice, the cell viability (CFU, colony forming units) is measured accordingly to the known methods from the state in the art. In practice, serial dilutions of a sample comprising live microorganisms are performed and plated onto an agar containing nutrient medium. CFU are counted from the applicable dilution(s).

- Survival before/after spray drying or freeze drying

In some other embodiments, the probiotic powder may be characterized by the survival of probiotics after spray drying or freeze drying.

The survival of probiotics after spray drying or freeze drying is calculated as follows:

$$\text{Survival} = N_d / N_0 \times 100\%,$$

wherein N_d refers to the probiotic population (CFU.g⁻¹) in powders after spray drying or freeze drying, while the initial population N_0 (CFU.g⁻¹) is calculated from N_+ and the total solid content (TS) of medium as following:

$$N_0 = N_+ \times (1 - \text{TS}) / \text{TS}.$$

The log reduction of bacteria after 120 days storage is calculated as follows:

$$\text{Log reduction} = \text{Log } N_d - \text{Log } N_{120},$$

wherein the N_{120} means the bacteria population in the powders when storage for 120 days.

In some embodiments, the probiotic powder may be characterized by a ratio $\text{Survival (TS 15-35\%)} / \text{Survival (TS } \leq 5\% \text{)} \geq 1.5$, wherein:

- “Survival (TS 15-35%)” represents a survival measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content ranging from 15 to 35 wt%, and

- “Survival (TS $\leq 5\%$)” represents a survival measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content equal or below 5 wt%.

In some embodiments, the ratio $\text{Survival (TS 15-35\%)} / \text{Survival (TS } \leq 5\% \text{)}$ is ranging from 1.5 to 50, which includes 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.4, 2.6, 2.8, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 and 48.

In some preferred embodiments, the ratio $\text{Survival (TS 15-35\%)} / \text{Survival (TS } \leq 5\% \text{)}$ is ranging from 1.5 to 25.

In some embodiments, the probiotic powder may be characterized by a ratio $\text{Survival (TS 15-35\%)} / \text{Survival (TS } \leq (5+10-30\% \text{)} \geq 1.5$, wherein:

- "Survival (TS 15-35%)" represents a survival measured from a probiotic powder obtained from a probiotic biomass cultured in a whey-containing nutrient medium having a total solid content ranging from 15 to 35 wt%, and

5 - "Survival (TS \leq (5+10-30%))" represents a survival measured from a probiotic powder obtained from a probiotic biomass cultured in a whey-containing nutrient medium having a total solid content of 5 wt%, and which total solid content was adjusted to 15 wt% or 35 wt% with freshly added whey after the growth of said probiotics is achieved.

10 In some embodiments, the ratio Survival (TS 15-35%)/Survival (TS \leq (5+10-30%)) is ranging from 1.5 to 10, which includes 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.4, 2.6, 2.8, 3, 4, 5, 6, 7, 8 and 9.

 In some preferred embodiments, the ratio Survival (TS 15-35%)/Survival (TS \leq (5+10-30%)) is ranging from 2 to 6.

Use

15 In another aspect, the invention relates to a probiotic powder according to the invention for use for improving health of a human or an animal body.

 In some embodiments, the probiotic powder is intended to be provided for human consumption.

20 In some embodiments, the probiotic powder may be provided for pets, such as dogs, cats, mice, rats, guinea pigs; horse; for economically important livestock, such as poultry, cattle, sheep, lambs, goats, pigs, struthios and aquaculture animals. As used herein, poultry encompasses chickens, hens, geese, turkeys, and quails.

25 The invention also relates to the use of a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, for culturing at least one probiotic bacterium, for the preparation of a probiotic powder comprising the said at least one probiotic bacterium.

 In some embodiment, a probiotic powder may be administered by oral route.

30 These compositions can be provided in the form of dissolved solutions or suspensions, tablets, coated tablets, capsules, syrups and the like. These compositions are prepared according to the usual methods in the food industry. The active ingredient can be incorporated into excipients normally used in these compositions, such as aqueous or non-

aqueous carriers, talc, arabic gum, lactose, starch, magnesium stearate, cocoa butter, fatty substances of animal or plant origin, paraffin derivatives, glycols, various wetting agents, dispersants or emulsifiers, or preserving agents.

For oral use of a probiotic powder according to the invention, the use of an
5 ingestible support is preferred. The ingestible support may be of diverse nature depending on the type of composition under consideration.

In some embodiments, the probiotic powder may be incorporated into a food product or a diet supplement.

A diet supplement may be formulated as milk or milk-based fermented
10 products, products based on fermented cereals, milk-based powders, food products of candy type, chocolate, cereals, tablets, gel capsules or lozenges, oral supplements in dry form and oral supplements in liquid form are especially suitable for use as food supports.

In some embodiments, the probiotic powder according to the invention may be used in the preparation of dairy food products, such as, e.g. milk products, fermented milk
15 products, yogurt, cheese, quark, chocolate mousse, frozen fermented dairy desserts, sour cream, and ice cream.

In some embodiments, the probiotic powder according to the invention may be used in the preparation of non-dairy food products, such as, e.g. vegetable-based drinks, peanut milk, fruit juices, soy-based products, cereal-based products, meat-based products
20 and the like.

In some embodiments, the probiotic powder according to the invention may be used in the preparation of vegetable-based feed, e.g. maize silage for animal feeding. Probiotics can be inoculated to the raw material and take part to the fermentation process.

In some other embodiments, the probiotic powder according to the invention
25 may be used in the preparation of veterinary compositions to be externally applied to the animal body, such as veterinary compositions suitable for being sprayed on the animal body. According to these embodiments, the probiotic bacteria contained in the probiotic powder according to the invention are aimed at colonizing the animal's skin.

In some embodiments, the probiotic powder according to the invention may be
30 used in the preparation of powders for prophylaxis against bacterial infections from environmental origin. Probiotics powder can be inoculated to the litter of farm animals

(e.g. cattle litter in barns) or of other, colonize the litter and avoid thus colonization by undesirable microorganisms (e.g. bacteria responsible for environmental mastitis).

In still other embodiments, the probiotic powder according to the invention may be used in the preparation of veterinary compositions or compositions for human use to be orally administered by inhalation, e.g. intranasally. According to these embodiments, the probiotic bacteria contained in the probiotic powder according to the invention are aimed at colonizing the respiratory tract of the administered human individual or of the administered animal.

A probiotic powder according to the invention may moreover be formulated with excipients and components that are commonly used for such oral compositions or food supplements, as for example, fatty and/or aqueous components, humectants, thickeners, preserving agents, texture agents, taste agents and/or coating agents, antioxidants, preserving agents and dyes that are common in the food industry.

In some embodiments, a probiotic powder according to the invention may be administered to a human or non-human body once, twice or three times a day, for one, two, three, four, five or six days, or one, two, three or four weeks, or one, two, three, four, five, six, or more months.

The dosage regimen of administration will be adapted by the skilled man according to the usual parameters taken into account in the field for setting a regimen of administration, such as, for example, the weight, the size, the age and/or the gender of the body.

In some embodiments, the probiotic powder comprising at least 10^9 CFU.g⁻¹ of at least one probiotic bacterium may be administered at a dose ranging from 0.01 mg to 10000 mg per day, preferably from 1 mg to 1000 mg per day.

In some embodiments, a dose ranging from 0.01 mg to 10000 mg per day includes a dose of 0.02 mg, 0.03 mg, 0.04 mg, 0.05 mg, 0.06 mg, 0.07 mg, 0.08 mg, 0.09 mg, 0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1000 mg, 2000 mg, 3000 mg, 4000 mg, 5000 mg, 6000 mg, 7000 mg, 8000 mg and 9000 mg per day.

In some embodiments, the probiotic powder comprising at least 10^9 CFU.g⁻¹ of at least one probiotic bacterium, may be mixed in a liquid and administered at a dose ranging from 0.01 mg.L⁻¹ to 10000 mg.L⁻¹ per day, preferably from 1 mg.L⁻¹ to 1000 mg.L⁻¹ per day.

In some embodiments, a dose ranging from 0.01 mg.L⁻¹ to 10000 mg.L⁻¹ per day includes a dose of 0.02 mg.L⁻¹, 0.03 mg.L⁻¹, 0.04 mg.L⁻¹, 0.05 mg.L⁻¹, 0.06 mg.L⁻¹, 0.07 mg.L⁻¹, 0.08 mg.L⁻¹, 0.09 mg.L⁻¹, 0.1 mg.L⁻¹, 0.2 mg.L⁻¹, 0.3 mg.L⁻¹, 0.4 mg.L⁻¹, 0.5 mg.L⁻¹, 0.6 mg.L⁻¹, 0.7 mg.L⁻¹, 0.8 mg.L⁻¹, 0.9 mg.L⁻¹, 1 mg.L⁻¹, 2 mg.L⁻¹, 3 mg.L⁻¹, 4 mg.L⁻¹, 5 mg.L⁻¹, 6 mg.L⁻¹, 7 mg.L⁻¹, 8 mg.L⁻¹, 9 mg.L⁻¹, 10 mg.L⁻¹, 20 mg.L⁻¹, 30 mg.L⁻¹, 40 mg.L⁻¹, 50 mg.L⁻¹, 60 mg.L⁻¹, 70 mg.L⁻¹, 80 mg.L⁻¹, 90 mg.L⁻¹, 100 mg.L⁻¹, 200 mg.L⁻¹, 300 mg.L⁻¹, 400 mg.L⁻¹, 500 mg.L⁻¹, 600 mg.L⁻¹, 700 mg.L⁻¹, 800 mg.L⁻¹, 900 mg.L⁻¹, 1000 mg.L⁻¹, 2000 mg.L⁻¹, 3000 mg.L⁻¹, 4000 mg.L⁻¹, 5000 mg.L⁻¹, 6000 mg.L⁻¹, 7000 mg.L⁻¹, 8000 mg.L⁻¹ and 9000 mg.L⁻¹ per day.

As shown in the example section below, the use of a two-in-one nutrient medium according to the invention allows:

- performing a two-step spray drying of a probiotic biomass composition comprising the steps of (i) growing a biomass of probiotics and (ii) spray drying said biomass composition in order to obtain a probiotic powder;
- increasing the viability of the probiotics of said probiotic powder;
- increasing the survival of said probiotics after spray drying;
- increasing the viability and the survival of the probiotics from said probiotic powder after long term storage.

The present invention is further illustrated, without in any way being limited to, the examples below.

Examples

Example 1

1. Material and methods

1.1. Strains and culture condition

The probiotic strain *Lactobacillus casei* BL23 was provided by UMR1219 MICALIS, (INRA-AgroParisTech, Jouy-En-Josas, France) and *Propionibacterium freudenreichii* ITG P20 was kept and pre-cultured by the CIRM-BIA Biological Resource

Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France). *L. casei* was activated by inoculating (1% inoculum size) in MRS Broth and cultivating statically at 37 °C for 16 h, while the *P. freudenreichii* was inoculated (1% inoculum size) in YEL broth and cultivated statically at 30 °C for 50 h.

5 1.2. Growth in sweet whey media

The sweet whey powder (Lactalis ingredients, Mayenne, France) was used to prepare the sweet whey media with different TS in this work. The composition of sweet whey powder was analyzed by the procedures described by (Gaucher et al., 2008)(Table 1).

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Table 1: Physical and chemical analysis of the sweet whey powder (the composition is expressed as (w/w)%, i.e. weight of component / weight of total solid content).

Total solids	94.81
pH	6.52
Total nitrogen (%)	11.81
Non-protein nitrogen (%)	3.11
Lactose (%)	67.91
Ashes (%)	6.85
Calcium (%)	0.37
Magnesium (%)	0.11
Sodium (%)	0.59
Potassium (%)	2.27
Chloride (%)	1.72
Phosphate (%)	1.14
Citrate (%)	2.26

15 The sweet whey powder was dissolved in deionized water to obtain media with the final total solids (TS, w/w) at 5%, 10%, 20%, 30% and 40% respectively. Additionally, the media with casein peptone were prepared by adding the casein peptone plus (Organotechnie, France) in the above sweet whey media at the concentration of 0.5% w/w (Cousin et al., 2012). These media were autoclaved at 100 °C for 30 min before inoculation of probiotic bacteria. The *L. casei* was inoculated at 1% inoculum size in the

different sweet whey media (i.e. with different TS of sweet whey and with/without casein peptone) with the MRS preculture. The inoculated media were incubated statically at 37 °C for 48 h. Similarly, *P. freudenreichii* was inoculated to the sweet whey media from YEL preculture and incubated statically at 30 °C for 120 h (5 days).

5

1.3. Spray drying

The probiotic culture grown in the casein peptone-supplemented media were used for spray drying. In addition to dry these bacteria culture directly, another group of samples were prepared by growing bacteria in 5% casein peptone-supplemented medium but increasing the TS value to 30% by adding the sweet whey powder before drying.

Spray drying was carried out with a pilot-scale Mobile MinorTM spray dryer (GEA Niro A/S, Denmark) with the maximal evaporation rate at 5 kg water h⁻¹. A two-fluid spray nozzle with the orifice diameter 0.8 mm was used in couple with a peristaltic pump (Watson-Marlow, France) for feeding and atomization. The humidity of inlet air was controlled at around 1g.kg⁻¹ of water in the air by a dehumidifier (Munters, Sweden). Spray drying parameters were monitored by the SD²P® software (Schuck et al., 2009). All the sample-contacted parts in the dryer including the nozzle, chamber etc. were washed by hot water at 90 °C. The 200 °C inlet temperature was used to dry the dryer and inactivate the possible microorganisms 2 h before drying the probiotic culture.

All the media (1 L) were agitated moderately 10 min before spray drying. Considering the intrinsic resistance of both strains, the inlet temperature of spray drying was set at 140 °C for *L. casei* (fragile strain) and 180 °C for *P. freudenreichii* (robust strain). The outlet temperature was 63 ± 2 °C for *L. casei* and 73 ± 2 °C for *P. freudenreichii*, and the outlet air relative humidity was controlled at 10 ± 1% by adjusting the feeding rate. The water content and water activity of the powders were tested according to the methods described by (Schuck et al., 2012).

1.4. Size distribution measurement and scanning electron microscopy

The size distribution of powders was measured by a laser light scattering with the MasterSizer 2000 equipped with a 5-mW helium-neon laser (Malvern Instruments, UK). The dry powder feeder attachment was used in couple with the standard optical model presentation for dispersion of powders in the air. The parameters $D_{0.5}$ and $Span$

were used to characterize the size distribution of powders, in which $D_{0.5}$ means that 50% of the particles had diameters smaller than this criterion, while *Span* of powders was calculated as:

$$Span = (D_{0.9} - D_{0.1}) / D_{0.5} \quad (1)$$

- 5 wherein $D_{0.9}$ and $D_{0.1}$ mean the criterion of diameters which 90% and 10% of the particles were smaller than respectively.

The result was from the mean of 2 independent samplings which had 3 successive measurements each time.

- 10 The powders of *L. casei* from the media with 5% and 30% TS were fixed on the carbon tape and then sputter-coated with the gold-palladium. These powder samples were observed by a scanning electron microscopy (JSM 7100F, JOEL, USA) at 5 kV (Fu et al., 2013).

1.5. Storage

- 15 The powders were collected and sealed in the sterilized polystyrene bottles (Gosselin, France). The sample contained bottles were stored under a controlled temperature of 4 °C and kept away from light. The samples were analyzed in a 30 day interval for 120 days (4 months).

20 1.6. Enumeration and quantification of bacteria viability

- The number of viable cells (CFU) was firstly measured after growth (i.e. before spray drying). The bacteria culture after 10 min agitation was diluted serially (1 mL to 9 mL) in peptone water (0.1% w/v). The powder samples after spray drying or during storage were rehydrated by dissolving 1 g powder in 9 mL peptone water before serial
25 dilutions. The diluted sample of *L. casei* was poured into MRS agar and incubated at 37 °C for 48 h (aerobic condition), while the dilution of *P. freudenreichii* was poured into YEL agar and incubated at 30 °C for 6 days under anaerobic condition (Anaerocult®, Merck KgaA, Germany).

- 30 The dependency of bacteria growth on the supplement of casein peptone was calculated as following equation:

$$Dependency = (N^+ - N^-) / N^- \quad (2)$$

wherein N^+ means the bacteria population (CFU.mL⁻¹) in the medium with supplement of casein peptone, N^- means without supplement of casein peptone.

The survival of bacteria after spray drying was calculated as:

$$\text{Survival} = N_d / N_0 \times 100\% \quad (3)$$

- 5 wherein N_d refers to the bacterial population (CFU.g⁻¹) in powders after spray drying, while the initial population N_0 (CFU.g⁻¹) was calculated from N^+ and the total solid content (TS) of medium as following:

$$N_0 = N^+ \times (1 - \text{TS}) / \text{TS} \quad (4)$$

The log reduction of bacteria after 120 days storage was calculated as

$$10 \quad \text{Log reduction} = \text{Log } N_d - \text{Log } N_{120} \quad (5)$$

wherein the N_{120} means the bacteria population in the powders when storage for 120 days.

1.7. Statistical analysis

- 15 All the experiments were repeated at least three times. The results were presented as mean value with standard error. Significant differences ($p < 0.05$) between the mean values were determined by Tukey's test. The statistical analysis was carried out using R 3.2.1 with the package of 'Rcmdr' (the R Development Core Team).

2. Results and Discussion

20 2.1. Growth of probiotics in sweet whey

- The growth of bacteria was compared in different nutrient media constituted by different TS of sweet whey, with or without supplement of casein peptone. As shown in the Figure 1A, the final population of *L. casei* in sweet whey media without casein peptone increased as TS increased from 5% to 40%. Indeed, the supplement of casein peptone significantly enhanced the final *L. casei* population from the TS of 5% to 30%, but not for that of 40%. The dependency of *L. casei* growth on casein peptone decreased when improving the TS of sweet whey. In these casein peptone supplemented media, the final *L. casei* population approximately 2×10^9 CFU.mL⁻¹ was reached for media with 20% and 30% TS, i.e. slightly higher than that obtained in MRS broth. The final population of *P. freudenreichii* also showed a similar trend when increasing the TS of sweet whey (Figure 1B). Specifically, the population also increased as the TS increased from 5% to 30%, but with a large reduction at 40%. The optimal TS values were located ranging from

20% to 30% as well, with higher populations than that obtained in YEL broth ($\sim 1.0 \times 10^9$ CFU.mL⁻¹). The casein peptone supplemented medium with 30% TS produced the highest population of *P. freudenreichii* ($\sim 2.5 \times 10^9$ CFU.mL⁻¹) among all the tested media in this work. However, compared to *L. casei*, the growth of *P. freudenreichii* in sweet whey media was less dependent on casein peptone. The maximal improvement was obtained when adding casein peptone in sweet whey with 20% TS. However, this dependency was around 1.0, which means that only one time improvement of *P. freudenreichii* population was found after the supplement of casein peptone.

The results indicate that increasing the TS of sweet whey to the range between 20% and 30% was able to improve the biomass production of both probiotic strains. As mentioned before, these higher TS values will be beneficial for the following spray drying. The improvement in the final bacteria populations may be caused by the richer nutrients in the media with higher TS value. Although the osmotic pressure was also higher in these media, the time of growth may be long enough to trigger the stress response of these two strains to adapt the high-osmolality environments (Wood, 2011). For example, it has been reported that both of these two strains were able to accumulate the intracellular polyphosphate which relates to the improvement of bacteria stress tolerance (Alcántara et al., 2014; Thierry et al., 2011). The presence of large amount of phosphate in the media with 20% or 30% TS may facilitate the accumulation of polyphosphate by uptake of phosphate from extracellular environment. However, when increasing the TS of sweet whey to 40%, the inhibition effect caused by the hyper osmolality and energy consumption in osmoregulation started to be deleterious for growth of the two strains.

2.2. Spray drying of probiotic cultured in sweet whey

The remaining viability of probiotics was expressed as the population and survival of bacteria after spray drying. The water content and water activity values of all powders were $6 \pm 1\%$ and $0.2 \pm 0.05\%$ respectively. As shown in Figure 2A, the survival of *L. casei* in powders after spray drying increased as the TS increased from 5% to 40%, with a maximal survival at around 40% for the TS values at 30% and 40%. In comparison with the powder from the medium of 5% TS, the survival in the powder from the medium of 30% TS was improved by approximately 60 folds ($\sim 0.6\%$ survival for 5% TS and $\sim 40\%$ survival for 30% TS). Correspondingly, the population of *L. casei* in powders also

increased as the TS increased from 5% to 30%, but with a reduction at that of 40% TS due to the relatively low population of *L. casei* in the medium of 40% TS before drying. For the strain of *P. freudenreichii*, the maximal survival (i.e. 70%) after spray drying appeared at the media with 20% and 30% TS. Similarly with that of *L. casei*, the media with low TS resulted in a lower survival at around 40% after spray drying. The population of viable bacteria in the powders from media with 5%, 10%, 20% and 30% were all higher than the level of 10^9 CFU.g⁻¹. The highest population was found in the medium of 20% TS, which reached the level of 10^{10} CFU.g⁻¹. The *P. freudenreichii* growing in medium with 40% TS showed the lowest survival and remaining population after spray drying.

When comparing between two probiotic strains, the survival of *P. freudenreichii* was generally higher than that of *L. casei* after spray drying, albeit the higher drying temperature being used. It implied that the *P. freudenreichii* strain was more tolerated during spray drying than the *L. casei* strain. It is known that *P. freudenreichii* is general heat resistant species which were often used for making Emmental-type cheeses in which the curd is heated at 50°C ~ 55 °C (Fox et al., 2004; Thierry et al., 2011). It has been described that *P. freudenreichii* accumulates the intracellular trehalose and glycogen in addition to polyphosphate, which involve in improving the bacterial tolerance against heat and/or desiccated stresses (Boyaval et al., 1999; Falentin et al., 2010).

To consider both the survival and final viable population of the probiotics in powders, the optimal TS value of sweet whey media was located between 20% and 30%. In this range, the two strains also presented the optimal biomass production. The improvement of survival may be caused by the robust cellular tolerance induced by higher osmolality in the media with TS from 20% to 30%. However, it could also be due to the higher dry matter (i.e. TS values). The relatively fine powders would be formed from the media with low TS due to the lower viscosity and less solid content within the particles, which may result in the longer retention time within the dryer (Jeantet et al., 2008). Besides, the lower solid content in medium also indicates that the less amount of wall material can be used to encapsulate the bacteria cells. In other words, the bacteria in the medium with lower TS may be exposed to the hot air to a larger extent (Perdana et al., 2014). Therefore, the lower solid content in the feeds seems to be disadvantageous for production powders with live probiotic bacteria.

In order to investigate the contribution of increased TS on the improved survival of probiotics after spray drying, a drying experiment was conducted by growing bacteria in 5% TS medium but increasing the TS to 30% immediately before drying (Figure 3C). After increasing the TS, the survival of *L. casei* after spray drying was improved to around 6% in comparison with that of growing and drying at 5% TS. However, it is still significantly lower than that of growing and drying at 30% TS. In contrast, the survival of *P. freudenreichii* decreased slightly (from 45% to 26%) when improving TS from 5% to 30% before drying, while growth in 30% TS significantly enhanced the spray drying survival to 70%.

The results indicated that the improvement of the probiotic survival after spray drying was not caused or only caused by the higher TS. The enhanced bacterial intrinsic tolerance triggered by high osmolality during growth may play the decisive role in the remaining of higher survival after spray drying. It has been reported that the salt-adaptation of *Lactobacillus paracasei* strain with high osmolality before spray drying (by 0.3 M NaCl) could improve the bacteria survival after heat treatment and spray drying (Desmond et al., 2001). In this work, the two bacteria strains were exposed to the high osmolality during the growth time (2 days for *L. casei* and 5 days for *P. freudenreichii*). It is known that reaching cellular homeostasis by accumulation of compatible solutes is an energy-dependent bacterial osmoregulation process (Guchte et al., 2002; Wood, 2011). In other words, the induction time is required for bacteria to adapt the adverse environments or trigger the stress response. In contrast to the bacteria growing in normal osmolality but exposed to the osmolality before drying, growing bacteria in the medium with high osmolality may have more enough time to accumulate the intracellular compatible solutes either by synthesis *per se* or uptake from environment (Figure 3).

2.3. Size distribution and morphology of powders

The size distribution of powders is shown in Figure 3 and Table 2.

Table 2. The size distribution of spray dried powders from media with different TS

	5%	10%	20%	30%	40%
<i>L. casei</i>					
$D_{0.5}$ (μm)	6.1 ± 0.1	6.9 ± 0.1	8.5 ± 0.1	15.3 ± 0.5	17.1 ± 0.1
Span	1.1 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
<i>P. freudenreichii</i>					
$D_{0.5}$ (μm)	7.2 ± 0.1	8.2 ± 0.1	10.0 ± 0.1	10.7 ± 0.1	17.6 ± 0.1
Span	1.4 ± 0.1	1.5 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	2.6 ± 0.1

For both strains, the size distribution of powders right shifted as the increasing of TS in the media. This result also corresponded to the observation by scanning electron microscopy. The powders dried from the media with high TS at 30% showed the larger span of size distribution ($Span > 2$), but with the $D_{0.5}$ larger than 10 μm . The powders from 5% medium were finer, with the narrow size distribution ($Span$ between 1 and 1.5) peaking below 10 μm . However, it was difficult to find the bacteria cells on the surface of powders in the SEM observation, even for the powders obtained from the medium with 5% TS. It indicated that the sweet whey with the 5% TS was already sufficient as the wall materials for microencapsulation of bacteria in spray drying. It coincided with the observation of spray dried powders of *Lactobacillus plantarum* with trehalose as drying medium (Perdana et al., 2014).

2.4. Viability of probiotics during storage

The changes of bacteria population in powders were monitored during storage at 4 °C for 120 days (Figure 4). For both *L. casei* and *P. freudenreichii* strains, the viabilities in the powders dried from the media with TS 20% ~ 40% remained constant with a maximal 0.89 log reduction. After storage for 120 days, *L. casei* population in the powders from 20% and 30% media remained the highest population at around 7×10^8 CFU.g⁻¹, while the *P. freudenreichii* at around 2×10^9 CFU.g⁻¹. Since the initial bacteria populations in the powders from 40% medium were relatively low, the *L. casei* and *P. freudenreichii* populations were at around 4×10^7 and 1×10^7 CFU.g⁻¹ respectively after 120 days. In comparison, the viable *L. casei* population in the powder from 5% medium significantly dropped during the entire storage time, with a final population below 10^6

CFU.g⁻¹ at the end of storage (~ 2.8 log reduction at 120 days). Meanwhile, the *L. casei* in the powder from 10% medium remained unchanged during the first 90 days (viability loss < 1 log), but displayed a large reduction after 90 days (~ 2.2 log reduction at 120 days). The powders of *P. freudenreichii* from the 5% and 10% media exhibited a considerably steady viability during the first 60 days. Although there was reduction of viability after storage afterward (~1.5 and 1 log reduction for 5% and 10% respectively), the final viable bacteria population still retained above 10⁸ CFU.g⁻¹ at the end of 120 days. The ability of *P. freudenreichii* to accumulate polyphosphate, trehalose and glycogen as energy and carbon storage compounds, as well as compatible solutes, could allow better survival throughout the long-term storage (Boyaval et al., 1999; Cardoso et al., 2007; Thierry et al., 2011).

Example 2

1. Material and methods

1.1. Strains and growth in sweet whey media

The probiotic bacteria used herein were provided by the Centre International de Ressources Microbiennes- Bactéries d'Intérêt Alimentaire (CIRM-BIA, Rennes, France). They are depicted in Table 3 below:

Strain	Source
<i>Bifidobacterium longum</i>	CIRM-BIA 1336
<i>Lactobacillus acidophilus</i>	CIRM-BIA 1674
<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	CIRM-BIA 1666
<i>Lactobacillus plantarum</i>	CIRM-BIA 466
<i>Lactobacillus reuteri</i>	CIRM-BIA 522
<i>Lactobacillus rhamnosus</i>	CIRM-BIA 607

The probiotic lactobacilli strain were cultured in MRS medium, while the bifidobacteria strain was culture in MRS supplemented with 0.05% cysteine for 16 h (*B. longum* and *L. acidophilus* at 37°C, *L. bulgaricus* at 43°C, and other lactobacilli strains at 30°C).

The sweet whey powder (Lactalis ingredients) was dissolved in deionized water to obtain media with the final total solids (TS, w/w) at 5 wt% and 30 wt%, respectively. These media were autoclaved at 115°C for 15 min before inoculation of probiotic bacteria. The probiotic bacteria were inoculated at 4% inoculum size in the different sweet whey media or in MRS medium with the MRS preculture. The inoculated media were incubated statically at 30°C, 37°C or 43°C for 24 h (corresponding to the growth temperature above mentioned).

1.2. Heat treatment

Following the growth in sweet whey medium, the biomass composition is brought to a temperature of 60°C for 10 min. CFU.mL⁻¹ are measured prior and after the heat treatment as to determine the survival of the probiotic bacteria.

2. Results and discussion

Table 4. Population of bacteria (data presented as Log₁₀ CFU.mL⁻¹) after growth in different sweet whey-containing media (4% inoculum, 24 h culture)

	<i>B. longum</i>	<i>L. acidophilus</i>	<i>L. delbrueckii subsp. bulgaricus</i>	<i>L. plantarum</i>	<i>L. reuteri</i>	<i>L. rhamnosus</i>
MRS	9.44	9.08±0.18	8.26	9.81	8.46±0.23	9.59±0.01
5% whey¹	8.54±0.10	8.00±0.06	8.00±0.06	8.61±0.19	8.32±0.03	9.24±0.01
30% whey¹	6.00	8.00±0.06	7.65±0.49	9.00±0.21	8.07±0.10	9.31±0.10

¹ Sweet whey.

As can be seen from Table 4, *Bifidobacterium longum* and all *Lactobacillus species* that are assayed substantially grow in sweet whey-containing nutrient medium, at 5 wt% and 30 wt%.

Table 5. Survival of bacteria (data presented as Log₁₀ N/N₀) after heat treatment

	<i><u>L. acidophilus</u></i>	<i><u>L. plantarum</u></i>	<i><u>L. reuteri</u></i>	<i><u>L. rhamnosus</u></i>
<u>MRS</u>	-4.75±0.18	<-5.81	<-4.49	-4.85±0.06
<u>5% whey¹</u>	<-4.00	<-4.63	-0.79±0.03	<-5.24
<u>30% whey¹</u>	-3.01±0.01	-4.00±0.03	-0.56±0.10	-2.81±0.01

¹ Sweet whey.

After heat treatment, namely 60°C for 10 min, the sweet whey-containing nutrient medium at 30 wt% significantly allows an increased protection of the probiotic bacteria, as compared to the sweet whey-containing nutrient medium at 5 wt%. The heat treatment performed herein represents harsher conditions than the spray drying technique. Indeed, although the temperatures of the heat treatment are similar to the temperatures performed in the spray drying, the heat treatment induces an overall heat stress that is superior to the overall heat stress encountered in spray drying. Therefore, the resistance of the bacteria to the heat treatment indicates that these bacteria would eventually display a higher survival at the end of a spray drying step, as disclosed in the present description.

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CLAIMS

1. A method for preparing a probiotic powder comprising at least one probiotic bacterium, said method comprising:

5 a) providing a probiotic biomass composition comprising at least one probiotic bacterium resulting from the culture of said probiotic bacterium in a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium;

10 b) spray drying or freeze drying the said probiotic biomass provided at step a) so as to obtain the said probiotic powder.

2. The method according to claim 1, wherein said probiotic bacterium is selected in a group comprising *Bifidobacterium sp.*, *Lactobacillus sp.*, *Lactococcus sp.*, *Propionibacterium sp.*, *Streptococcus sp.* and a mixture thereof.

15 3. The method according to claim 1 or 2, wherein said probiotic bacterium is selected in a group comprising *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*, *Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. rhamnosus*, *Lactococcus lactis*, *Propionibacterium freidenreichii*, *Streptococcus thermophilus* and a mixture thereof.

20 4. The method according to any one of claims 1 to 3, wherein the whey-containing nutrient medium has a total solid content ranging from 26 % by weight to 33 % by weight, based on the total weight of the said whey-containing nutrient medium.

5. The method according to any one of claims 1 to 4, wherein, at step b), the inlet temperature of spray drying is ranging from 120°C to 200°C.

25 6. The method according to any one of claims 1 to 5, wherein, at step b), the outlet temperature of spray drying is ranging from 55°C to 80°C.

7. The method according to any one of claims 1 to 6, wherein said nutrient medium further comprises casein peptone as a preferred nitrogen source.

30 8. A probiotic powder comprising at least one probiotic bacterium, which probiotic powder is obtained by the method according to any one of claims 1 to 7.

9. The probiotic powder according to claim 8, wherein the particle size distribution is characterized by a ratio $D_{0.5} (TS \ 15-35\%) / D_{0.5} (TS \leq 5\%) > 1$, wherein:

- "D0.5(TS 15-35%)" represents a D0.5 particle size measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content ranging from 15 % by weight to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, and

- "D0.5(TS $\leq 5\%$)" represents a D0.5 particle size measured from a probiotic powder obtained from a probiotic biomass resulting from a culture of probiotics in a whey-containing nutrient medium having a total solid content equal or below 5 % by weight, based on the total weight of the said whey-containing nutrient medium.

10 10. The probiotic powder according to claim 9, wherein the particle size distribution is characterized by a ratio $D0.5(TS 15-35\%)/D0.5(TS \leq 5\%) > 1.3$.

11. The probiotic powder according to claim 10, wherein it comprises at least 10^8 CFU.g⁻¹ of the said at least one probiotic bacterium.

12. The probiotic powder according to any one of claims 8 to 11, for use for improving health of a human or an animal body.

13. The use of a whey-containing nutrient medium having a total solid content ranging from above 25 % by weight to up to 35 % by weight, based on the total weight of the said whey-containing nutrient medium, for culturing at least one probiotic bacterium, for the preparation of a probiotic powder comprising the said at least one probiotic bacterium.

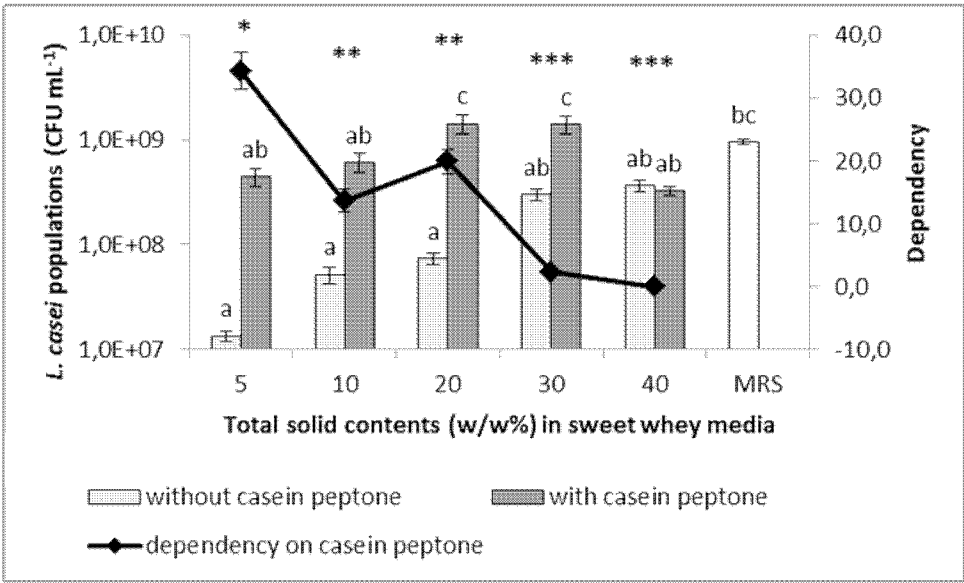


FIGURE 1A

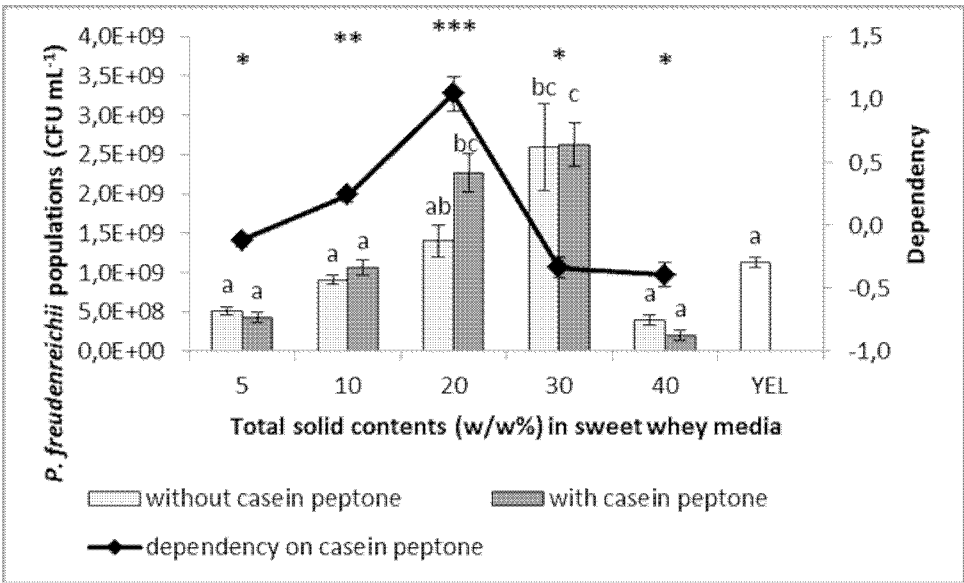


FIGURE 1B

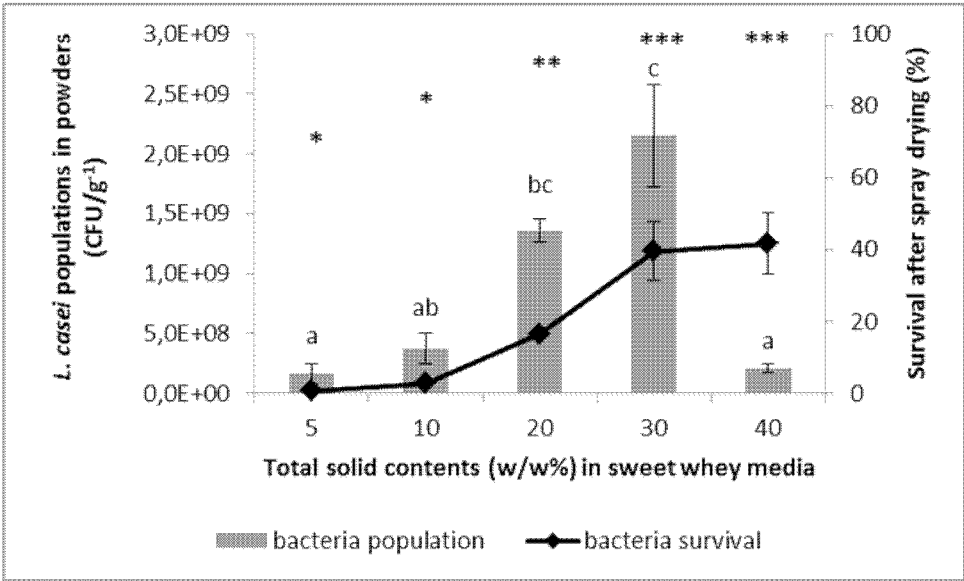


FIGURE 2A

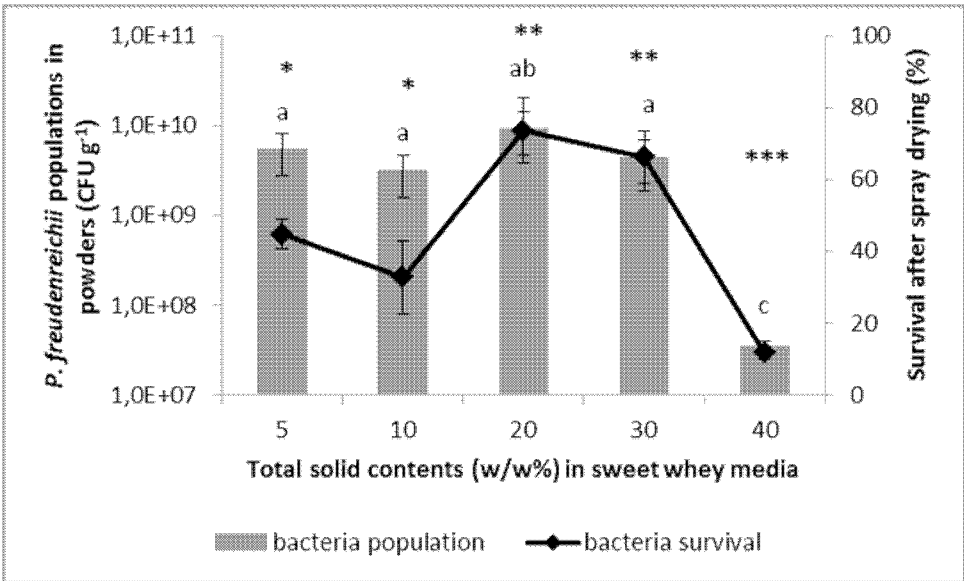


FIGURE 2B

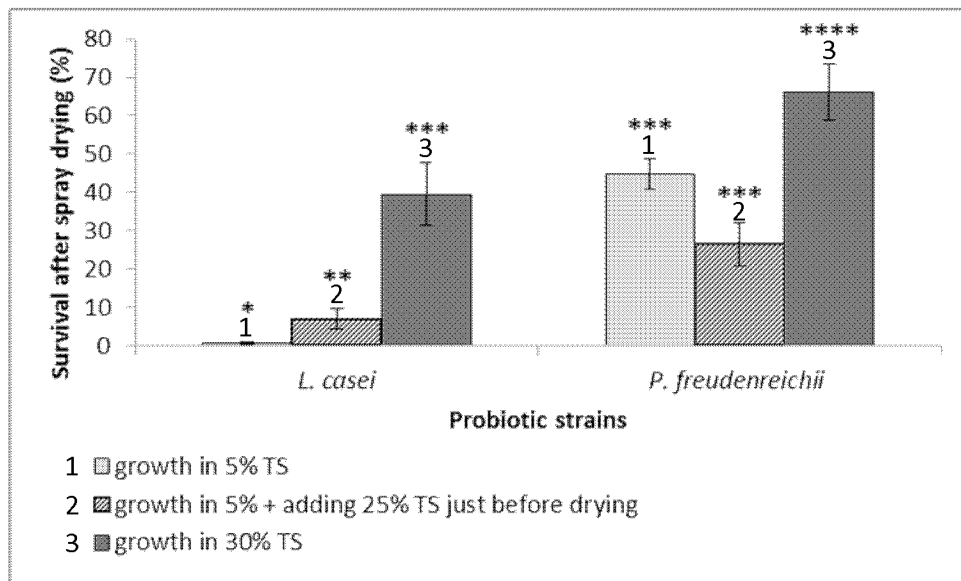


FIGURE 2C

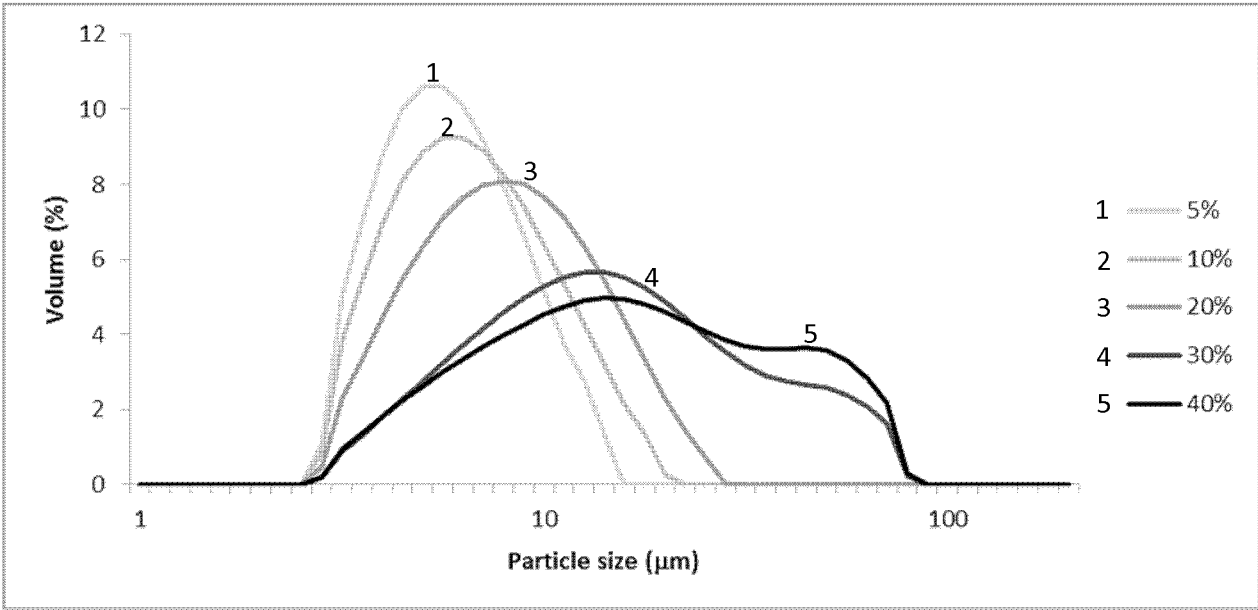


FIGURE 3A

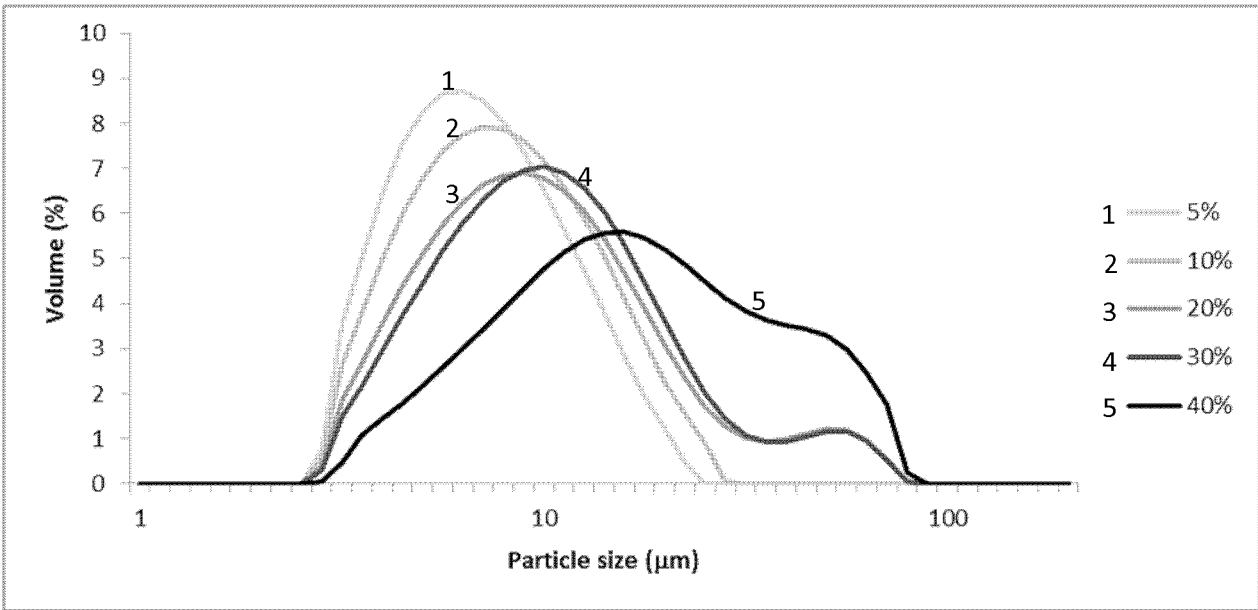


FIGURE 3B

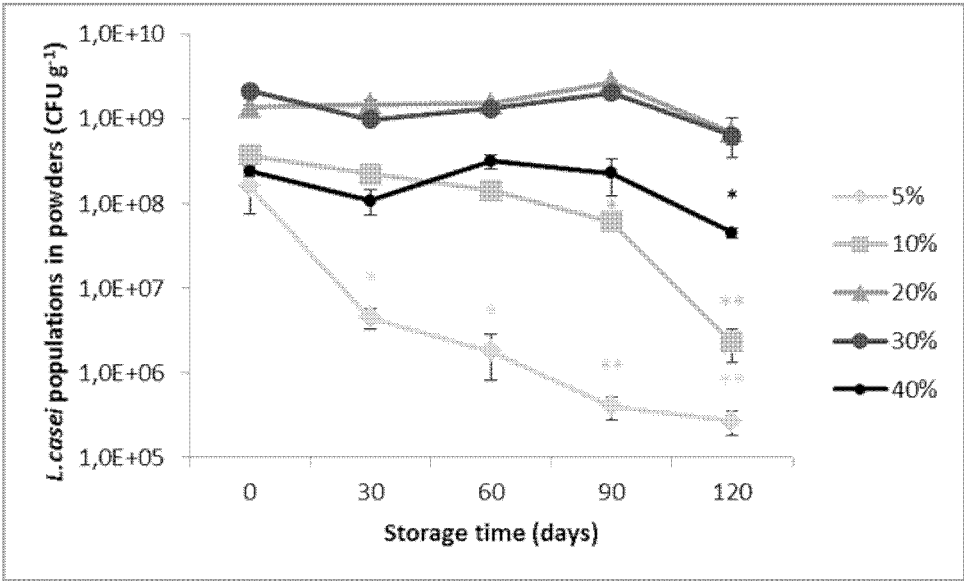


FIGURE 4A

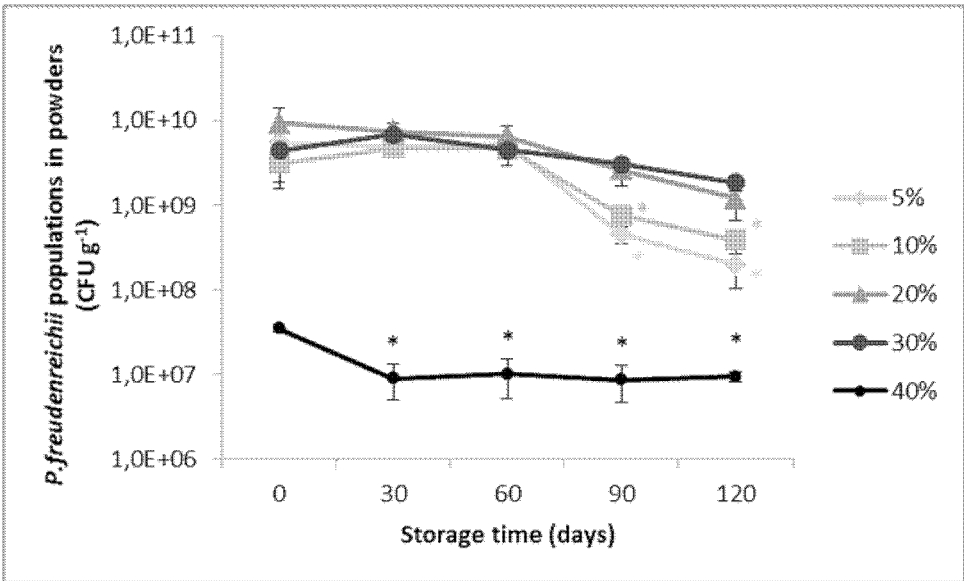


FIGURE 4B

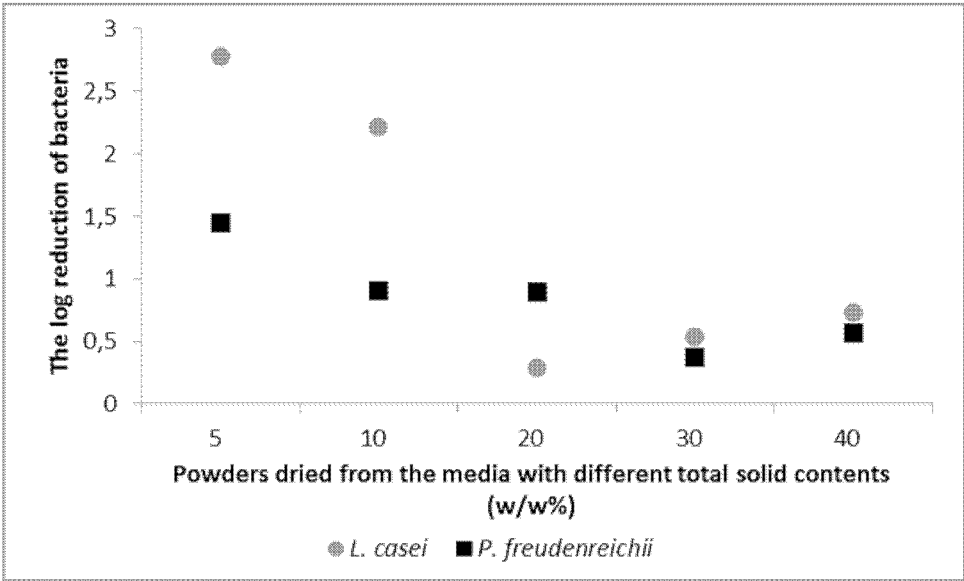


FIGURE 4C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/072324

A. CLASSIFICATION OF SUBJECT MATTER INV. A23L33/135 C12N1/04 A61K35/74 A23L33/195 A23L33/19 A23P10/40 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) A23L A61K C12N 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, WPI Data, FSTA, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 010 725 A (MEISTER NIKLAUS [CH] ET AL) 4 January 2000 (2000-01-04) examples 1,3,4 -----	1-13
X	RU 2010 140092 A (G OBRAZOVATEL NOE UCHREZHDENIE VYSSHEGO PROFESSIONAL NOGO OBRAZOVANIJA) 10 April 2012 (2012-04-10) abstract -----	1-13
X	US 4 423 079 A (KLINE LEO [US]) 27 December 1983 (1983-12-27) claims 1,7; examples 1,2 -----	1-13
X	CS 9 100 190 A3 (VYZK USTAV MLEKARENSKY [CS]) 12 August 1992 (1992-08-12) abstract -----	1-13
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<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search		Date of mailing of the international search report
24 November 2016		02/12/2016
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 Tallgren, Antti

INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>M. JANTZEN ET AL: "Direct spray drying and microencapsulation of probiotic Lactobacillus reuteri from slurry fermentation with whey", JOURNAL OF APPLIED MICROBIOLOGY., 1 July 2013 (2013-07-01), pages n/a-n/a, XP055254941, GB</p> <p>ISSN: 1364-5072, DOI: 10.1111/jam.12293</p> <p>page 1029, paragraphs 1,2</p> <p>page 1030, right-hand column, paragraph 2</p> <p>page 1033, left-hand column, paragraph 3 -</p> <p>page 1035, left-hand column, paragraph 2</p> <p>-----</p>	1-13

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