



(51) International Patent Classification:

A23C 9/123 (2006.01) C12N 1/38 (2006.01)
C12R 1/46 (2006.01) C12P 1/00 (2006.01)
C12N 1/04 (2006.01) C12R 1/225 (2006.01)
C12N 1/20 (2006.01)

(21) International Application Number:

PCT/EP2014/073505

(22) International Filing Date:

31 October 2014 (31.10.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

13191313.9 1 November 2013 (01.11.2013) 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:

- as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

[Continued on next page]

(54) Title: USE OF ALGAE TO INCREASE THE VIABLE ACTIVE BIOMASS OF LACTIC ACID BACTERIA

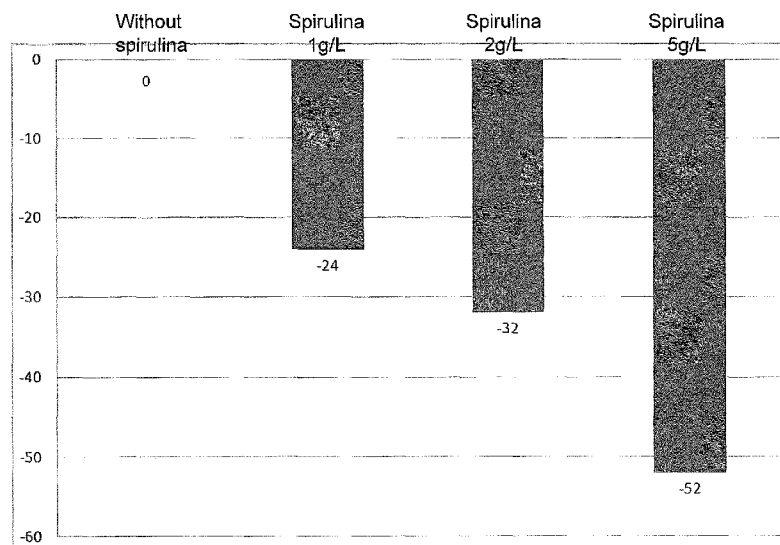


Fig. 1

(57) Abstract: A method of producing a lactic acid bacteria (LAB) composition, or harvested LAB composition, with increased LAB viable active biomass using algae or a fraction thereof is described. The algae may be prokaryotic algae, such as a cyanobacteria, or an eukaryotic algae selected from the group consisting of a red algae and a green algae, or any fraction thereof, and in particular is selected from the group consisting of spirulina, chlorella and a fraction thereof.



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))

USE OF ALGAE TO INCREASE THE VIABLE ACTIVE BIOMASS OF LACTIC ACID BACTERIA

TECHNICAL FIELD

The present invention relates to the field of bacterial culture, particularly the culture of lactic acid bacteria, and most particularly the culture of lactic acid bacteria under aerobic growth conditions and the use of algae to increase the viable active biomass of lactic acid bacteria under aerobic growth conditions, as well as LAB compositions and harvested LAB compositions produced in this way.

BACKGROUND

Lactic acid bacteria (LAB) are extensively cultured worldwide for use in the food and feed industry. They are used in the manufacture of fermented products including dairy, meat, bakery, wine and vegetal products. Consumption of fermented products has lately been increasing worldwide, and therefore there is a need to find ways to increase the active biomass of LAB produced by culture.

LAB biomass production can be achieved following two different metabolic pathways: aerobic and anaerobic. It has been described that in LAB, aerobic respiration is activated by exogenous heme or haem (Lechardeur *et al*: Current Opinion in Biotechnology 2011, 22:143-149). Hemin is an iron containing porphyrin compound, derived from red blood cells. In optimal aerobic conditions, the LAB cells are able to use oxygen to produce ATP with a higher efficiency that results in a higher biomass yield and a lower production of inhibitory lactate compared to anaerobic fermentation. Therefore additives such as hemin, which increase aerobic respiration and thus biomass of LAB in culture, are valuable in industry. Hemin for use in LAB culture has usually been sourced from porcine or bovine hemoglobin.

The Inventors have identified other cell growth activators, algae or fractions thereof, which can be used to increase growth of LAB under aerobic conditions.

Algae are photosynthetic organisms both unicellular and multicellular. Algae are mostly eukaryotic organisms and can be classified based on their pigmentation, and thus encompass red algae (Rhodophyta), brown algae (Heteromontophyta), green algae

(Chlorophyta) and Diatomaceae. The term "algae" also encompasses the prokaryotic algae, such as cyanobacteria informally referred to as blue-green algae.

Algae and extracts thereof are known to increase the growth of some LAB under anaerobic conditions (see for example Bhowmik *et al.* World Journal of Dairy & Food Sciences (2009), 4(2), pp160-163). Algae extracts are also known to influence the production of acid in milk under anaerobic conditions, which is known as the booster effect of algae on LAB (Molnár *et al.* Milchwissenschaft (2005), 60(4), pp380-382). However, the specific uses, methods and culture conditions of the inventions disclosed herein have not previously been described.

SUMMARY OF INVENTION

The present invention provides the use of an algae of a fraction thereof, in particular an algae selected from the group consisting of spirulina, chlorella and a fraction thereof, to increase under aerobic growth conditions the biomass, in particular the viable active biomass, of lactic acid bacteria (LAB). Furthermore, a method of producing a LAB composition and harvested LAB composition with increased viable active biomass is provided.

The present invention provides an increased biomass, in particular an increased viable and/or active biomass, of LAB in aerobic culture, through the use of algae and fractions thereof. In particular the present invention details the use of an algae or a fraction thereof selected from the group consisting of spirulina, chlorella and a fraction thereof, to increase under aerobic growth conditions the biomass, in particular the viable active biomass, of LAB. The present invention uses specific assays to identify and quantify the increase in biomass of LAB.

The present invention also provides LAB compositions and harvested LAB compositions, including intermediate LAB starter cultures, LAB starter cultures and probiotics, particularly intermediate LAB starter cultures and LAB starter cultures for use in producing fermented products.

SOME ADVANTAGES

The use of algae or a fraction thereof in culturing LAB increases the biomass, in

particular the viable active biomass, of LAB produced. Thus, the methods and uses of the present invention reduce cost associated with the production of LAB compositions and harvested LAB compositions. In addition or alternatively, the LAB compositions and harvested LAB compositions produced by the methods of the invention have a reduced lag phase (Ta value) and are therefore faster acidifiers.

The current invention provides a method of increasing LAB biomass as demonstrated by a reduced lag phase (Ta value) and/or measurable increase in the colony forming units (CFU) of the LAB composition or harvested LAB composition of the invention. In particular, the current invention provides at least an equivalent, or even an increased, LAB viable active biomass compared to the use of hemin instead of algae or a fraction thereof.

The current invention further provides a LAB composition, a harvested LAB composition, intermediate starter culture, starter culture or LAB probiotic composition.

Algae are a vegetal additive. Therefore the use of algae or a fraction thereof, instead of animal-derived hemin, allows consumers with diets such as vegetarian, vegan, kosher and Halal to consume the resulting products.

In addition, algae or a fraction thereof is easily produced and found worldwide, and therefore can be sourced relatively cheaply and easily.

DESCRIPTION OF THE FIGURES

The present invention will be described by reference to the following Figures:

Figure 1: Spirulina concentration impact on Ta activity (minutes) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

Figure 2: Chlorella concentration impact on Ta activity (minutes) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

Figure 3: Spirulina concentration impact on CFU concentration (CFU/ml) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

Figure 4: Chlorella concentration impact on CFU concentration (CFU/ml) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

Figure 5: Spirulina concentration impact on OD gain (delta OD) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

5 Figure 6: Chlorella concentration impact on OD gain (delta OD) of *Lactococcus lactis* DGCC2631 cultured under aerobic conditions

DETAILED DESCRIPTION

10 The present inventors have surprisingly found that some algae or a fraction thereof can be used to increase the biomass, in particular the viable active biomass, of lactic acid bacteria (LAB), under aerobic growth conditions.

The invention is directed to a method of producing a LAB composition with increased LAB viable active biomass comprising the steps of:

- 15 (i) admixing a culture medium and a LAB, and an algae or a fraction thereof;
- (ii) culturing said LAB admixture under aerobic growth conditions to produce said LAB composition;

wherein said increased viable active biomass is defined as at least one of the following:

- 20 a) the lag phase of growth on a fermentable substrate (Ta value), of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or
- 25 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof.

The invention is also directed to a method of producing a harvested LAB composition comprising the steps of:

- 30 (i) admixing a culture medium and a LAB, and an algae or a fraction thereof;

(ii) culturing said LAB admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

- 5 a) the lag phase of growth on a fermentable substrate (Ta value), of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or
- 10 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

(iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed
15 and/or by separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed, to produce a harvested LAB composition.

In a particular embodiment, the harvesting step is separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed

20 In a particular embodiment, the harvesting step is separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed. As a particular embodiment, the harvested LAB composition, when at least 80% of the final culture medium is removed, also has an increased lactic acid bacteria viable active biomass, wherein said increased viable active
25 biomass of the harvested LAB composition is defined as at least one of the following:

- 30 a) the lag phase of growth on a fermentable substrate (Ta value), of the harvested lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a harvested LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

- b) the content of viable cells, expressed in colony forming units (CFU), of the harvested lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a harvested LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof

Furthermore, the invention also provides for the use of an algae or a fraction thereof, in the manufacture of a lactic acid bacteria composition for increasing viable active biomass of LAB, cultured under aerobic growth conditions, wherein increased viable active biomass is defined as at least one of the following:

- a) wherein the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a LAB culture medium, or same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or
- b) wherein the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof.

Furthermore, the invention also provides for the use of an algae or a fraction thereof, in the manufacture of a harvested lactic acid bacteria composition for increasing viable active biomass of LAB, cultured under aerobic growth conditions, wherein increased viable active biomass is defined as at least one of the following:

- a) wherein the lag phase of growth on a fermentable substrate (Ta value) of the harvested lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a harvested LAB culture medium, or same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or
- b) wherein the content of viable cells, expressed in colony forming units (CFU), of the harvested lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a harvested LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof.

A "fermentable substrate" is a material that contains an organic compound such as a carbohydrate that can be transformed (i.e., converted into another compound) by the enzymatic action of a bacterium such as LAB. The term "substrate" also means a fermentable substrate.

For example, a fermentable substrate may consist of, contain or comprise milk, non-fat dry milk, vegetables (e.g., corn potatoes, cabbage), starch, grains (e.g. rice, wheat, barley, hops), fruit (e.g., grapes, apples, oranges), sugar, sugarcane, meat (e.g., beef, poultry, pork, sausage), heart infusion, cultured dextrose, combinations thereof, and media containing proteins, carbohydrates, and minerals necessary for optimal growth.

In a particular embodiment the fermentable substrate is milk, which may be referred to as "milk substrate".

The term "active", in the context of the present invention means lactic acid bacteria, and/or a culture or composition thereof, which are able to produce lactic acid after incubation or culture in a fermentable substrate (suitably milk), in order to decrease the pH. For example, to decrease the substrate pH of at least 0.1 pH unit. In a particular embodiment, the pH decrease is at least 0.1 and at most 3 pH units.

The term "viable" in the context of the invention means lactic acid bacteria which are able to grow and to multiply on an appropriate medium, for example a complex medium, a specified medium, an enriched medium, nutrient medium and/or Medium A of the examples. The medium may be liquid, liquid paste or solid, such as agar medium. In addition or alternatively, "viable" refers to LAB which contain cytoplasmic enzyme activity, identified by specific fluorochromes and/or by appropriate staining and/or which present cell membrane integrity, for example identified by direct epifluorescent filter technique (DEFT – see for example Bunthof CJ, Bloemen K, Breeuwer P, Rombouts FM, Abee T. 2001. Flow cytometric assessment of viability of lactic acid bacteria. Appl Environ Microbiol 67:2326-2335. In addition Comas-Riu J, Rius N. 2009. Flow cytometry applications in the food industry. J Ind Microbiol Biotechnol 36:999-1011.) or flow cytometry methodologies. Viable LAB may also be referred to as "viable cells".

The aim of the use or method of the invention is to increase the viable active biomass of LAB and/or to produce a LAB composition and/or a harvested LAB composition with increased LAB viable active biomass, wherein "active" and "viable" are defined as above.

- 5 The terms "medium", "media" and "culture medium" mean a composition, which may be liquid, gelatinous or solid, containing nutrients, which may include carbon, nitrogen, minerals, vitamins and/or fat sources, preferably at an optimum concentration, in which LAB can grow and multiply. The culture medium is not milk. In a particular embodiment, the culture medium is not skim milk, skimmed milk, milk-containing medium, skim milk-
10 containing medium, skimmed milk-containing medium, reduced fat milk or 0.1% milk. A "LAB culture medium" is separate and is defined in more detail below.

In a particular embodiment, the medium may comprise a carbohydrate, a yeast or yeast extract, buffer, surfactant, vitamin and/or nutrient sources (such as a vitamin C source),
15 defoamer (or antifoamer, or anti-foamer) and a solvent (suitably water).

In a particular embodiment, the medium may comprise lactose, a yeast extract, magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), Manganese(II) sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), Na ascorbate, defoamer and soft water (water with a reduced content of
20 cations and anions).

In a particular embodiment, the culture medium is the medium A as defined in point 1.3 of the examples below.

- 25 A medium may also be referred to as a "nutrient medium", "nutrient media", "growth media" or "nutrient and growth media/medium".

In one embodiment the medium is not MRS.

- 30 The terms "culturing" as used herein means any method to grow and to multiply microbial organisms, such as LAB, by enabling them to reproduce in predetermined culture medium under controlled conditions of pH, temperature oxygen content, stirring and length of growing time (also known as culture length). The term "culture" may be used to refer to the culturing of any microbial organisms, such as one or more LAB

strains as exemplified below. Within the present invention, culturing is aerobic or under aerobic growth conditions as defined below.

1. The term “admixing” as used herein means mixing together to combine or form one
5 substance. In one embodiment, in the method of the invention the term “admixing” refers to mixing a culture medium, lactic acid bacteria and an algae or a fraction thereof. The mixing may be carried out in any order. For instance, in one embodiment the culture medium may be mixed with the algae or a fraction thereof prior to mixing this combination with a lactic acid bacteria. The combination of the algae or a fraction thereof
10 and the culture medium may be optionally sterilised prior to adding the lactic acid bacteria. Components which have been admixed may be referred to herein as an admixture or mixture.

The phrase “aerobic growth conditions” refers to growth requiring the presence of
15 oxygen, preferably dissolved oxygen, in the culture medium or the LAB culture medium. The phrases “aerobic growth conditions” and “aerobic conditions” are used interchangeably.

Aerobic growth conditions may be achieved by continuous aeration, e.g. continuous air
20 flow and/or agitation (e.g. stirring, rotating and/or shaking).

Aerobic growth conditions may for example be obtained by continuous agitation, such as rotating and/or stirring and/or shaking, at normal air atmosphere (e.g. 1 atm). For example agitation (e.g. stirring, rotating and/or shaking) may be carried out at 10-
25 1000rpm, 100-500rpm, 100-300rpm, 100-200 rpm or 100-200 rpm. In one embodiment agitation occurs at 160rpm. In another embodiment, agitation occurs at 30-150rpm, preferably 50-100rpm. Alternatively, aerobic growth conditions can be achieved by a continuous air flow, for example by using bubbling, or by air circulation such as by using an airlift such as in an air lift-type fermentor. Dissolved Oxygen (O_2) is preferably
30 controlled at a value of 10-30%, suitably at a value of 15-25% and most preferably at around 20% or 20%.

Dissolved O_2 may be maintained at the required value for example by using a probe for monitoring, such as using a pO₂ probe for monitoring. The dissolved O_2 in the culture

media may be controlled by the regulation of the air injection flow rate, such as by using a air sparger or microsparger, and/or by bubbling air at the bottom of the fermentor wherein the LAB is cultured. In one embodiment an air flow rate from 0.3vvm-5vvm, suitably 1-4vvm, suitably 2-3vvm may be used.

5

Aeration may be carried out by continuous rotating and/or shaking and/or stirring. Typically, the energy an impeller or stirring device dissipates into the liquid/broth is in the range of 0.2 to 5 kW/m³, in particular 1 to 2 kW/m³.

10 In one embodiment the culturing is carried out in a fermentor, e.g. a fermenting vat.

In one embodiment the culturing is carried out in an Erlenmeyer flask.

15 In a particular embodiment, large fermentors (industrial scale) are used, with an air flow rate at 0.3vvm to 5vvm, and the culture is stirred with an energy dissipation of the impeller between 0.2 to 5 kW/m³, in particular 1 to 2 kW/m³, to produce aerobic growth conditions.

20 In another embodiment a 2L fermentor, with an air flow rate at 0.3vvm to 5vvm, and stirring from 500 to 1000 rpm may be used.

25 In another embodiment using a 1L Erlenmeyer, rotating and/or shaking, at normal air atmosphere between 100-500rpm, 100-300rpm, 100-200 rpm or 100-200 rpm may be used. In one embodiment shaking may occur at 160rpm. This may be considered equivalent to 0.3vvm to 5vvm using an impeller for stirring, such as a Rushton impeller type, stirring from 200 rpm to maximum of 1000rpm.

30 Foam formation in the medium may be controlled by the admixing, preferably injection of, appropriate defoamer or anti-foamer. Preferably such injection may be carried out when a conductivity sensor is in contact with the foam. Foam formation may also be controlled using a mechanical foam breaker in one embodiment.

In one embodiment, culturing the LAB is carried out in a controlled pH environment. Suitably the pH of the medium is therefore controlled at or around 5-7, most suitably pH 6-7 and in one embodiment pH is controlled at 6.2.

- 5 In one embodiment, culturing the LAB (in the method or use of the invention) is carried out at a controlled temperature. Suitably the temperature is from 10-50°C, most suitably 20-45°C, most suitably the temperature is 25-35 °C and more suitably culturing is carried out at a temperature of 30 °C or at around 30 °C, such as 26, 27, 28, 29, 30, 31, 32, 33 or 34 °C .

10

Culturing is carried out for at least 1-30 hours, most suitably 4-24 hours, more suitably 10-20 hours, more suitably 15-20 hours, more suitably 16-18 hours and most suitably for at or around 18 hours.

- 15 In a particular embodiment, the culture is stopped when the LAB in the LAB composition have reached a sufficiently high concentration, in particular have reached at least a concentration of 10^8 to 10^{12} CFU / ml of the LAB composition, most suitably at least 10^9 , at least 10^{10} , at least 10^{11} , at least 10^{12} CFU / ml of the LAB composition.

- 20 In a preferred embodiment the increase in LAB in the LAB composition during culturing is between 10,000 to 10,000,000 fold. Most preferably the increase in LAB during culturing is between 10,000 to 1,000,000 fold.

- 25 In a particular embodiment, during culturing, the LAB in the LAB composition increases (e.g. from about 10^4 CFU / ml of culture medium at the start of culturing) to 10^8 to 10^{12} CFU / ml of the LAB composition (at the end of the culturing).

- 30 In one embodiment, the LAB in the LAB composition increases to at least 10^9 CFU / ml of the LAB composition (at the end of the culturing), e.g. at least 10^{10} , at least 10^{11} CFU / ml of the LAB composition.

In a particular embodiment, aerobic growth conditions according to the invention are as follows: in a 1L Erlenmeyer flask, at least 100 ml of culture medium, comprising the

algae or fraction thereof at the desired concentration and admixed with a selected LAB, incubated for 18h at 30°C on a shaker at 160 rpm (for example an INFORS shaker).

5 The uses and methods of the invention comprise culturing at least one strain of lactic acid bacteria under aerobic conditions in an appropriate nutrient and growth medium, at an appropriate temperature, in which at least one algae or a fraction thereof is present or is added.

10 In the uses or methods of the invention defined herein, the parameter "*T_a*" is the time in minutes for the LAB composition or harvested LAB composition to decrease the pH of a fermentable substrate by 0.08 pH units. Preferably the fermentable substrate is milk. *T_a* is the time taken for the lag phase of growth. Thus, the lower the *T_a*, the shorter the lag phase.

15 In a preferred embodiment of the current invention, the *T_a* value is determined after the initial culture of LAB under aerobic growth conditions. In other words, a sample or starter culture obtained from the LAB composition (from step (ii) of the method) is grown on a fermentable substrate in order to determine the *T_a* value.

20 The term "*T_a* with algae" or "*T_a* algae" may be used to indicate the *T_a* of a LAB composition or harvested LAB composition or a sample thereof, cultured under aerobic growth conditions in the presence of algae or a fraction thereof. "*T_a* control" is the *T_a* of the LAB culture medium or the harvested LAB culture medium, which has been cultured under identical aerobic growth conditions, and harvested by identical harvesting
25 methods if the LAB composition under the determine "*T_a* algae" was a harvested LAB composition. In other words, it is the *T_a* of an identical culture of LAB or same sized sample thereof, wherein the algae or a fraction thereof has been excluded from the culturing process. The only difference is that the control does not include algae or a fraction thereof in the culture.

30

The calculation "*T_a* with algae minus *T_a* control" is the difference in minutes between the *T_a* of a LAB composition or harvested LAB composition or a sample thereof (after culture under aerobic conditions in presence of the algae or a fraction thereof) and the *T_a* of the LAB culture medium or the harvested LAB culture medium (which is the same LAB

composition or same harvested LAB composition or same sized sample thereof after culture under the same aerobic conditions in absence of the algae or a fraction thereof) which is the control. A reduction in the Ta value may be symbolised by a minus value. For example “-10” indicates a reduction in Ta of 10 minutes.

5

Preferably the Ta value is determined using Assay I as described herein.

The difference between the Ta algae and the Ta control of at least 10 minutes means that the addition of the algae or a fraction thereof produced a LAB composition or harvested LAB composition after culture which decreases the lag phase in milk, i.e., that the LAB of the composition after culture in presence of algae or a fraction thereof are more active in milk. In a particular embodiment, the Ta value difference between the Ta algae and the Ta control is at least 10 minutes, at least 12 minutes, at least 15 minutes, at least 20 minutes, at least 30 minutes or at least 40 minutes.

15

It is noteworthy that the results achieved by the present invention are not due to the addition of algae or a fraction thereof to the fermentable substrate (e.g. milk). The LAB in the composition or harvested LAB composition of the present invention are more active (as measured by Ta decrease in milk) than identical LAB cultured in identical conditions without algae or a fraction thereof (see example 2.3). The effects achieved with the present invention are over and beyond those which would be achieved by addition of algae (or a fraction thereof) alone to the milk. The effect of the algae (or a fraction thereof) on the LAB in the LAB composition or harvested LAB composition of the invention is to increase activity compared to addition of algae (or a fraction thereof) alone to milk, and therefore are not solely due to known booster effect.

20
25

If the LAB composition is a harvested LAB composition, to determine the calculation “Ta with algae minus Ta control” as described above, it should be compared to a LAB culture medium or a same sized sample thereof (obtained after culture in absence of the algae (or a fraction thereof) and used as a control) which has undergone the same harvesting methods.

30

In the use or method defined herein, the parameter “CFU/ml” qualifies the concentration of viable biomass obtained in the LAB composition or harvested LAB composition and is

expressed as CFU/ml (colony forming units) after enumeration on appropriate media such as agar. The higher the CFU count per ml of LAB composition or harvested LAB composition, the higher the viable biomass concentration of the LAB. The calculation “ratio CFU with algae / CFU control” is the ratio (expressed in %) of the CFU of the LAB composition or harvested LAB composition of the invention compared to the LAB culture medium or harvested LAB culture medium or a same sized sample thereof (obtained after culture in absence of the algae (or a fraction thereof) -control).

If the LAB composition is a harvested LAB composition, to determine the ratio “CFU with algae / CFU control” as described above, it should be compared to a LAB culture medium or a same sized sample thereof (obtained after culture in absence of the algae (or a fraction thereof) and used as a CFU control) which has undergone the same harvesting methods.

Preferably CFU is determined using Assay II as described herein.

A ratio “CFU with algae / CFU control” of at least 120% means that the addition of the algae (or a fraction thereof) produced a LAB composition or harvested LAB composition after culture which contains more viable cells. In a particular embodiment, the ratio “CFU with algae / CFU control” is of at least 130, at least 140, at least 150 or at least 200 %, i.e., the content of viable cells of a lactic acid bacteria composition obtained after culture in the presence of said algae (or a fraction thereof) under aerobic conditions, is at least 130, at least 140, at least 150 or at least 200 % the content of viable cells of a lactic acid bacteria culture medium or a harvested lactic acid bacteria culture medium obtained after culture in the absence of said algae (or a fraction thereof).

Algae

The term “algae” as used herein refers to any algae including the prokaryotic algae and the eukaryotic algae, and encompasses also any fraction of algae, as defined in this application.

In a particular embodiment, the algae used in the invention is a prokaryotic algae, in particular a cyanobacteria, or a fraction thereof. In a particular embodiment, said

cynaobacteria is *Spirulina*, also known as *Arthrospira* (*Arthrospira* species), such as *Arthrospira platensis* (or *Spirulina platensis*,) or *Arthrospira maxima*. Herein, *Spirulina* may also be referred to as "Spiruline".

5 In a particular embodiment, the algae (or a fraction thereof) used in the invention is an eukaryotic algae, in particular a red algae, or a green algae or a fraction thereof. In a particular embodiment, the algae used in the invention is an eukaryotic algae selected from the red algae or the green algae. In a particular embodiment, the algae used in the invention is a green algae, in particular an algae from the Chlorophyta division, in particular from the Chlorellales order, in particular from the Chlorellaceae family, and in particular is a *Chlorella* species. *Chlorella* encompasses species such as *Chlorella vulgaris*. It is well known as a health food (see for Example Becker, E.W. Biotechnol Adv. 2007 Mar-Apr;25(2):207-10). In a particular embodiment, the algae used in the invention are red algae, in particular an algae from the Rhodophyta division.

15 In a particular embodiment, when the algae (or a fraction thereof) is an eukaryotic algae or is selected from an eukaryotic algae and a prokaryotic algae, such as a cyanobacteria, said algae is not a brown algae, in particular is not an algae from the Phaeophyceae class. In particular, the algae is not an algae from the Laminariales Order, in particular is not an algae from the Laminariaceae Family, in particular is not a *Laminaria* species (laminaire), such as *Laminaria digitata*. In another embodiment, the algae is not an algae from the Fucales order, in particular is not an algae from the Fucaceae family, in particular is not a *Fucus* species, such as *Fucus vesiculosus*.

25 In a particular embodiment, the algae (or a fraction thereof) is a prokaryotic algae, in particular a cyanobacteria, or eukaryotic algae selected from the group consisting of a red algae and a green algae, or any fraction thereof.

30 In a particular embodiment, the algae (or a fraction thereof) used in the invention are selected from the group consisting of *Spirulina*, *Chlorella* or a fraction thereof.

The algae used in the invention may include any fraction of said algae.

The phrase "*a fraction thereof*" when applied to algae refers to an extract, suspension, dilution, part, fragment of algae, such as spirulina fraction or chlorella fraction. The fraction may be in dried, powdered, liquid, diluted, reconstituted powder suspension or other reconstituted form. It may also be a fresh extraction from growing algae such as growing spirulina or chlorella. The fraction may include all active parts of the algae.

In one embodiment the fraction of algae is in the form of whole algae, powdered, and in a further embodiment the fraction of algae is in the form of whole algae, dried and powdered. The term "dried" preferably means less than or equal to 8% humidity. The term "powdered" preferably refers to a particle size of less 300 µm mean diameter, preferably less than 250, 200, 190, 180, 170, 160, 150 140, 130, 120, 100, 90 or 80µm mean diameter. Preferably the algae or fraction thereof is reconstituted in a liquid before use, preferably said liquid is water. The algae may be commercially sourced.

An "extract" may refer to a liquid, aqueous, powdered or dried extract of any part of the algae. A "suspension" may refer to any part or extraction of algae suspended in a liquid. A "dilution" may refer to any part or any extraction, such as a liquid extraction, diluted with any liquid. A "part" of an algae may refer to any section of algae including green parts, blade stipe, stem or holdfast.

Non limiting examples of spirulina fractions which may be used in the invention include, but is not limited to, Spirulina extract from the following providers: Spirulina from Setalg, Spirulina from SEAH, Spirulina from COPCI or Spirulina from AlphaBiotec Miette. A non limiting example which may be used in the invention includes chlorella extract from Setalg.

The algae or fractions thereof used in the described invention may be heat treated.

In a preferred embodiment the algae or a fraction thereof is sterilised before use in the culture medium of the invention. Any suitable form of sterilisation may be used. By way of example only, the sterilisation may be a heat treatment. Alternatively or in addition, the sterilisation may comprise a chemical treatment and/or a radiation treatment

In one embodiment the algae or a fraction thereof may be sterilised before it is added to the medium.

Alternatively or in addition, the algae or a fraction thereof may be added to the culture
5 medium and the combination of culture medium and algae (or a fraction thereof) may be sterilised before addition of the LAB and culturing.

Sterilisation may be carried out by heat treatment. In one embodiment the sterilisation may be carried out by heating the sample to 100°C or more. In a preferred embodiment
10 sterilisation of the algae or a fraction thereof, or algae (or a fraction thereof) and culture medium, is carried out at 100-130°C.

Sterilisation preferably is carried out for at least 5 minutes. Suitably the sterilisation may be carried out for 10-30 minutes.

15

In one embodiment sterilisation of the algae or a fraction thereof, or algae (or a fraction thereof) and culture medium, is carried out at 100-130°C for 10-30 minutes.

In one embodiment sterilisation of the algae or a fraction thereof, or algae (or a fraction thereof) and culture medium, occurs at 115-125°C for 15-25 minutes.
20

In a preferred embodiment sterilisation of the algae, or algae and culture medium, occurs at 121°C for 20 minutes.

25 In the use or method defined herein, the algae, in particular spirulina, chlorella or a fraction thereof, is used, is present or is added to the culture medium in which the LAB composition is cultured under aerobic growth conditions. In a particular embodiment of any use or method of the invention, said algae, in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium, at a concentration of at
30 least 0.1 g/L, at least 0.2 g/L, at least 0.3 g/L, at least 0.4 g/L, at least at least 0.5 g/L, in particular at least 1 g/L.

Concentrations of algae are given herein as gram (g) of algae or a fraction thereof by litre (L) of culture medium, before inoculation of a culture medium with LAB. In a

particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration of at least 2 g/L. In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration of at least 5 g/L. Said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, can be present or be added to the culture medium at a concentration as high as 20, 30, 40, 50, 60, 70, 80, 90 or 100 g/L. In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium from a concentration selected from the group consisting of 0.1, 0.5, 1 and 2 g/L to a concentration selected from the group consisting of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 0.1 to 10 g/L.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 0.5 to 10 g/L.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 1 to 10 g/L.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 1 to 5 g/L.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 2 to 10 g/L.

In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium at a concentration from 5 to 10 g/L.

- 5 In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is present or is added to the culture medium before inoculation of the LAB into said culture medium.

- 10 In a particular embodiment, said algae (or a fraction thereof), in particular spirulina, chlorella or a fraction thereof, is added to the culture medium after inoculation of the LAB into said culture medium, but before culturing said LAB composition.

Lactic Acid Bacteria (LAB)

- 15 In a particular embodiment of any use or method of the invention, the term "lactic acid bacteria" (LAB) refers to any bacteria which produce lactic acid as the end product of carbohydrate fermentation. In a particular embodiment, the LAB is selected from the group consisting of species *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Bifidobacterium* or any combination thereof.

20

By "LAB composition", it is meant a composition comprising at least one LAB strain, or a defined composition comprising two or more LAB strains or an undefined composition of LAB strains, which has been treated in accordance with the present invention to increase viable active biomass.

25

By "harvested LAB composition" it is meant a LAB composition as defined herein which has further undergone harvesting methods as described herein. In a particular embodiment, said harvested LAB composition is obtained after separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed.

30

In a particular embodiment, said harvested LAB composition is obtained after separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed.

In a particular embodiment, the LAB composition or harvested LAB composition comprises a pure LAB strain, or a defined composition of pure LAB strains or an undefined composition of pure LAB strains. In a particular embodiment of the uses or the methods of the invention, the LAB composition or harvested LAB composition consists of
5 a strain from species *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* or *Bifidobacterium*, or comprises at least a strain from species *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* or *Bifidobacterium*.

10 In a particular embodiment of the uses or the methods of the invention, the LAB composition or harvested LAB composition consists of a strain from species *Lactococcus* or a strain from species *Leuconostoc*, or comprises at least a strain from species *Lactococcus* or at least a strain from species *Leuconostoc*. In a particular embodiment of the uses or the methods of the invention, the LAB composition or harvested LAB
15 composition consists of a strain from species *Streptococcus thermophilus* or comprises at least a strain from *Streptococcus thermophilus*. By “at least one strain” or “at least a strain”, it is meant 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 strains, or even more.

In particular, the LAB composition or harvested LAB composition comprises a strain of
20 species *Lactococcus*, in particular a strain of *Lactococcus lactis*, in particular a strain of *Lactococcus lactis ssp. lactis* or a strain of *Lactococcus lactis ssp. cremoris*. In a particular embodiment, the LAB composition or harvested LAB composition is a mixture of more than one LAB for example of a) at least one strain of species *Lactococcus*, in particular a strain of *Lactococcus lactis*, in particular a strain of *Lactococcus lactis ssp.*
25 *lactis* or a strain of *Lactococcus lactis ssp. cremoris*, and b) at least one strain selected from the group consisting of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium* and any combination thereof.

In a particular embodiment, the LAB composition or harvested LAB composition consists
30 of several strains of the species *Lactococcus*, in particular of several strains of *Lactococcus lactis*, in particular of several strains of *Lactococcus lactis ssp. lactis* or of several strains of *Lactococcus lactis ssp. cremoris*. By “several strains”, it is meant at least 2, in particular 2, 3, 4, 5, 6, 7, 8, 9 or 10 strains, or even more. In a particular embodiment, the LAB composition or harvested LAB composition consists of at least one

strain, in particular one strain or several strains, of *Lactococcus lactis* ssp. *lactis* and at least one strain, in particular one strain or several strains, *Lactocococcus lactis* ssp *cremoris*. In a particular embodiment, the LAB composition or harvested LAB composition consists of a strain of species *Lactococcus*, in particular a strain of

5 *Lactococcus lactis*, in particular a strain of *Lactococcus lactis* ssp. *lactis* or a strain of *Lactocococcus lactis* ssp *cremoris*. In a particular embodiment, the LAB composition or harvested LAB composition comprises or consists of the *Lactococcus lactis* strain DGCC2631 (deposited at DSMZ under accession number DSM27777) and/or the *Lactococcus lactis* strain DGCC9209 (deposited at DSMZ, under accession

10 No.DSM12015).

In a particular embodiment, the LAB composition or harvested LAB composition comprises or consists of a strain of species *Leuconostoc*, in particular *Leuconostoc mesenteroides*. In a particular embodiment, the LAB composition or harvested LAB

15 composition comprises or consists of several strains of the species *Leuconostoc*. In a particular embodiment, the LAB composition or harvested LAB composition is a mix of a) at least one strain of species *Leuconostoc*, in particular a strain of *Leuconostoc mesenteroides*, and b) at least one strain selected from the group consisting of *Lactobacillus*, *Pediococcus*, *Bifidobacterium* and any combination thereof. In a particular

20 embodiment, the LAB composition comprises or consists of the *Leuconostoc mesenteroides* strain DGCC 4634.

Any LAB composition or harvested LAB composition as described herein within the invention may also comprise additional microorganism(s), prokaryotic or eukaryotic, such

25 as yeast, mould or other bacteria, for example *Corynebacterium* species, *Staphylococcus* species.

A LAB composition or harvested LAB composition of the invention may also comprise remaining algae or a fraction thereof, and/or the culture media.

It is noteworthy that when a LAB composition or harvested LAB composition comprises or consists of several LAB strains, or LAB strain(s) and additional microorganism(s), the parameters defined herein (Ta, OD gain or CFU) are determined for the whole LAB composition after culture, or for the whole harvested LAB composition.

A sample of a LAB composition or a harvested LAB composition is any fraction of the LAB composition or harvested LAB composition. For example, 0.1%, 0.2%, 0.5% or 1% weight by weight of the composition. It may be dried and or powdered, and/or liquid
5 and/or concentrated.

A "LAB culture medium" is a LAB admixed with a culture medium to form a LAB composition which is defined as a composition *not* treated with algae (or a fraction thereof) in accordance with the present invention.

10 A "harvested LAB culture medium" is a LAB admixed with a culture medium to form a LAB composition which is defined as a composition *not* treated with algae (or a fraction thereof) in accordance with the present invention, which has been cultured under aerobic growth conditions and has further undergone harvesting methods as described
15 herein.

A LAB culture medium may comprise any LAB, and may for example comprise any of the LAB strains of the LAB composition. Where a LAB culture medium is a control, it is cultured under the same conditions as the LAB composition of the invention (in particular
20 regarding aerobic conditions), and comprises initially the same LAB strains, but is *cultured in the absence of algae*.

By "same sized sample" herein is meant a sample obtained in an identical way of the same weight and/or volume. When a LAB composition of the invention and a LAB
25 culture medium are compared by any test, a same-sized sample is used.

In a particular embodiment of the method or the use of the invention, the concentration of the LAB in the culture medium before culture or the concentration of LAB added to the culture medium before culture is in the range of 10^5 to 10^{10} CFU (colony forming units)
30 per ml of the culture medium.

Optical density

Optical density (OD) can be used to evaluate the biomass of a culture.

Optical density (OD) increase or “gain” between the beginning of a culture and the end of a culture is positively correlated to the cell biomass production. Thus, a high OD gain corresponds to a high bacterial cell concentration in the composition.

5

By “*OD gain of a LAB composition cultured in the presence of said algae under aerobic conditions*”, it is meant the difference between the optical density (OD) of a LAB composition after culture in the presence of said algae (or a fraction thereof) under aerobic conditions and the OD of the same LAB composition before culture in the presence of said algae [also called lactic acid bacteria admixture]. By “*OD gain of the LAB culture medium cultured in the absence of said algae*”, it is meant the difference between the OD of the LAB culture medium after culture in the absence of said algae (or a fraction thereof) under aerobic conditions minus the OD of the same LAB culture medium before culture in the absence of said algae (or a fraction thereof).

10

The calculation “ratio delta OD algae / delta OD control” (expressed in %) is the ratio of the OD gain as defined above for a LAB composition after culture under aerobic conditions in presence of the algae or a fraction thereof, over the OD gain as defined above for a lactic acid bacteria culture medium cultured under the same aerobic conditions in absence of algae or a fraction thereof (control).

15

The increased viable active biomass of the LAB composition of the invention may further be defined as an optical density (OD) gain of the LAB composition of at least 120% the OD gain of the LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae (or a fraction thereof). In a particular embodiment, said OD gain of a LAB composition of the invention, is at least 130, at least 140, at least 150, at least 200 or at least 300 % the OD gain of the LAB culture medium (cultured in the absence of said algae or a fraction thereof).

20

In a particular embodiment, the OD is determined at a wavelength of 400-800nm, suitably 500-700nm, most suitably 600-700nm. In a particular embodiment, the OD is determined at a wavelength of 660nm. OD may be determined using a spectrophotometer, for example using a Genesys 20™ Spectrophotometer (Thermo

25

Scientific). An example of assay to determine the delta OD of a culture is given in Assay III below.

Assay I (Ta measurement)

5

Assay I is a milk activity test, which may be used to determine the Ta of any cultured LAB. In this case it is used to determine the Ta of the LAB composition of the invention compared to the Ta of the LAB culture medium (control).

10 Milk acidifying activity of the biomass of the LAB composition obtained after culture under aerobic conditions [or the LAB culture medium - control] is qualified on CINAC equipment using a multi pH probe recorder. Thus, pH decrease is recorded using CINAC equipment from Alliance Instruments which is 32 channel pH probe recorder.

15 The milk substrate used is prepared by dissolving skim milk powder (for example skim milk from Humana) into deionised water at a 9/100 dilution (for example 90gr of skim milk powder into 910gr of water). Rehydration takes place for 30 minutes at room temperature under stirring conditions, and is followed by sterilising the reconstituted milk in 480ml volume at 96°C for 30min.

20

The milk substrate is pre warmed at 30°C and then inoculated at 0.1% weight by weight with a non pH control culture of a LAB composition [obtained after culture of LAB under aerobic growth conditions either in absence of algae or a fraction thereof (control), in presence of algae or a fraction thereof, or in presence of hemin]. For each milk activity
25 test, pH is recorded for 16h at 30°C.

For each test, Ta in minutes (min) is recorded, and corresponds to the time in minutes for the LAB composition after culture [or the LAB culture medium - control] to decrease the milk pH by 0.08 units. This time period is called lag phase.

30

This assay is as described in the international standard ISO/DIS 26323 IDF 313 2006 "Milk products - Determination of the acidification activity of dairy cultures by continuous pH measurement (CpH)".

Assay II (CFU measurement)

Assay II is used to calculate the CFU (number of colony forming units) of any cultured LAB. In this case it is used to determine the CFU of the LAB composition of the invention
5 compared to the CFU of the LAB culture medium (control).

1ml of LAB composition of the invention (obtained after culture under aerobic growth conditions in presence of algae or a fraction thereof), or 1ml of LAB culture medium (obtained after culture under aerobic growth conditions in absence of algae or a fraction
10 thereof - control), or in 1ml of LAB culture medium cultured in the additional presence of hemin, is diluted by mixing in 9ml of TS tryptone salt diluent at room temperature to obtain a dilution 10⁻¹ after mixing on a vortex for 30s; this dilution procedure is repeated by inoculation of 1 ml of dilution 10⁻¹ into 9 ml of TS diluent till 10⁻⁸. Proper inoculums of dilutions 10⁻⁷ and 10⁻⁸ are then plated on M17 agar using Eddy jet spiral inoculator's
15 and incubated under anaerobic conditions at 30°C for 48h. At the end of incubation, grown colonies are enumerated and CFU is determined after multiplication by the dilution factor.

This assay is as described in the international standard ISO 27205/ IDF 149 (2010) and
20 ISO 7889/IDF 117 (2003):15214 /ISO 15214:(1998) "Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of mesophilic lactic acid bacteria - Colony-count technique at 30 degrees C".

Assay III (Optical Density)

25

Assay III is used to calculate the optical density (OD) of any cultured LAB.

1ml of LAB composition of the invention (obtained after culture under aerobic growth conditions in presence of algae or a fraction thereof), or 1ml of LAB culture medium (obtained after culture under aerobic growth conditions in absence of algae or a fraction
30 thereof- control), or in 1ml of LAB culture medium cultured in the additional presence of hemin, is diluted into 9, 18 or 27 ml of distilled water in order to obtain a diluted sample, and an OD range comprised between 0.1 and 0.5 on diluted sample. The diluted sample is then measured at 660nm, using a Genesys 20™ spectrophotometer (with a spectral

slit width specification of 8nm and an effective optical path of 10mm); the OD (Optical Density) is calculated by multiplying the dilution factor of the bacterial culture by the value given by the spectrophotometer. Calculated OD is the multiplication of the dilution factor by the value given by the spectrophotometer.

5

For each sample in the examples disclosed herein, in absence of algae or a fraction thereof (LAB culture medium - control), in presence of algae or a fraction thereof (the LAB composition of the invention), or in presence of hemin, two OD are calculated: the OD of the composition before culture, and the OD of the composition after culture under aerobic conditions. Finally, the OD gain of each sample, corresponding to the difference between the OD of the sample after culture under aerobic conditions and the OD of the same sample before culture, is calculated.

10

Harvesting

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The lactic acid bacteria produced by any of the methods or uses herein described may be harvested. Thus, a LAB composition as defined herein which has further undergone harvesting methods as defined herein is a "*harvested LAB composition*".

20

Harvesting involves separating the LAB of the LAB composition from at least a percentage of the final culture medium, and/or from at least a portion the algae (or a fraction thereof).

25

In some embodiments the LAB may be isolated from the final culture medium and/or from algae (or a fraction thereof).

30

In a particular embodiment, the culture is stopped and harvesting occurs when the LAB concentration in the final culture medium (or LAB composition) reaches a sufficiently high concentration, in particular reaches a concentration of 10^8 to 10^{12} CFU / ml of the LAB composition, most suitably at least 10^9 , 10^{10} , 10^{11} or 10^{12} CFU/ml of the LAB composition.

In a particular embodiment harvesting is carried out using any conventional techniques enabling the separation of the LAB in the LAB composition from the final culture medium and/or the algae or a fraction thereof.

- 5 Suitably harvesting concentrates the LAB (and thus a harvested LAB concentrate (e.g. a harvested LAB composition concentrate) is obtained).

Harvesting and concentration may use, but is not limited to, any appropriate technology of sedimentation or flocculation, in particular by centrifugation and/or membrane filtration
10 and/or ultrafiltration. Alternatively, concentration may optionally be carried out as a separate, additional step.

In a particular embodiment, the harvesting step implements any conventional methods, including the ones cited above, in order to concentrate the harvested LAB. In a preferred
15 embodiment the LAB in the harvested LAB composition may reach a concentration of 10^8 to 10^{13} CFU, or 10^8 to 10^{12} CFU/g / of the harvested LAB composition.

In a preferred embodiment the LAB in the harvested LAB composition may reach a concentration of at least 10^8 , at least 10^9 , at least 10^{10} , at least 10^{11} or at least 10^{12}
20 CFU/g of the harvested LAB composition.

In a preferred embodiment the LAB are concentrated in the harvested LAB composition by a factor of 5-40 fold. The concentration factor is defined as the ratio between the resulting harvested LAB composition and the non-harvested LAB composition from
25 which it is harvested. In a preferred embodiment the LAB are concentrated by a factor of 10-30 fold. In a most preferred embodiment, the LAB are concentrated by a factor of 15-25 fold.

By "*final culture medium*", it is meant the water and water-soluble components of the culture medium at the end of the culture (i.e., when one decides to stop the growth of the
30 LAB). Water-soluble components include for example residual substrates and metabolites obtained following the culture of the LAB (such as for example lactate).

Harvesting produces a harvested LAB composition with lower amounts of final culture medium and/or algae, and a higher concentration of LAB.

In a particular embodiment, the harvesting step comprises or consists of separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed, to produce the harvested LAB composition. In a particular embodiment, at least 85% of the final culture medium is removed. In a particular embodiment, at least 90% of the final culture medium is removed. In a particular embodiment, at least 95% of the final culture medium is removed. This produces a harvested LAB composition with a higher concentration of LAB compared with the LAB composition.

The final culture medium may be separated from the LAB composition to produce the harvested LAB composition, using any conventional techniques including in particular by centrifugation and/or filtration, and/or membrane filtration and/or ultrafiltration. Harvesting and concentrating may also involve purifying. Purifying may involve removing the colour, particularly the green colour, of the algae (or a fraction thereof), from the harvested LAB. This separation in one embodiment excludes drying.

Alternatively or in addition to the previous embodiment, the harvesting step comprises or consists of separating the LAB from the final culture medium, such as at least 5% of the algae or a fraction thereof present during culturing is removed, to produce the harvested LAB composition. Preferably at least 10% of the algae or a fraction thereof is removed. Preferably at least 20% of the algae or a fraction thereof is removed. Preferably at least 30% of the algae or a fraction thereof is removed. Preferably at least 40% of the algae or a fraction thereof is removed. Preferably at least 50% of the algae or a fraction thereof is removed. Preferably at least 60% of the algae or a fraction thereof is removed. Preferably at least 70% of the algae or a fraction thereof is removed. Preferably at least 80% of the algae or a fraction thereof is removed. Preferably at least 90% of the algae or a fraction thereof is removed. Preferably 100% of the algae or a fraction thereof is removed.

In one embodiment 5-100% of the algae or a fraction thereof is removed. In one embodiment 5-90% of the algae or a fraction thereof is removed. In one embodiment 5-80% of the algae or a fraction thereof is removed. In one embodiment 5-70% of the

algae or a fraction thereof is removed. In one embodiment 5-60% of the algae or a fraction thereof is removed. In one embodiment 5-50% of the algae or a fraction thereof is removed. In one embodiment 5-40% of the algae or a fraction thereof is removed. In one embodiment 5-30% of the algae or a fraction thereof is removed. In one embodiment 5-20% of the algae or a fraction thereof is removed. In one embodiment 5-10% of the algae or a fraction thereof is removed. In one embodiment 10-100% of the algae or a fraction thereof is removed. In one embodiment 10-90% of the algae or a fraction thereof is removed. In one embodiment 10-80% of the algae or a fraction thereof is removed. In one embodiment 10-70% of the algae or a fraction thereof is removed. In one embodiment 10-60% of the algae or a fraction thereof is removed. In one embodiment 10-50% of the algae or a fraction thereof is removed. In one embodiment 10-40% of the algae or a fraction thereof is removed. In one embodiment 10-30% of the algae or a fraction thereof is removed. In one embodiment 10-20% of the algae or a fraction thereof is removed.

The harvested LAB composition may comprise algae (or a fraction thereof), media and extracts of said algae (or a fraction thereof) and media. In one embodiment, the harvested LAB composition does not comprise all of the algae or a fraction thereof present in the final culture medium and/or LAB composition.

The harvested LAB composition is preferably a concentrated harvested LAB composition.

Algae (or a fraction thereof) may be removed from the LAB composition to produce the harvested LAB composition, using any conventional techniques including in particular by centrifugation and/or filtration, and/or membrane filtration and/or ultrafiltration. Harvesting and concentrating may also involve purifying. Purifying may involve removing the colour, particularly the green colour, of the algae (or a fraction thereof), from the harvested LAB.

The removal of the algae or fraction thereof may improve the quality of the product obtained following the addition into a fermentable substrate of said harvested LAB composition (or intermediate starter culture or starter culture as defined herein obtained from said harvested LAB composition).

By “improve the quality”, it is meant a reduction of the green color and/or an improvement in taste such as a reduction in bitterness, and/or a reduction in presence of insoluble extracts in said products.

- 5 Thus, the removal of the algae or fraction thereof may improve the quality of food products or feed products, in particular of the fermented products, in particular fermented food products or fermented feed products, in particular in particular dairy products, obtained using said harvested LAB composition (or intermediate starter culture or starter culture as defined herein obtained from said harvested LAB composition).

10

In a particular embodiment, after harvesting, the LAB, in particular the harvested LAB concentrate, is maintained as a liquid or in a liquid form.

Intermediate starter cultures

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The invention disclosed herein also includes LAB intermediate starter cultures, methods of producing said intermediate starter cultures and uses of said intermediate starter cultures.

- 20 By “*intermediate starter culture of the invention*”, as used herein it is meant one harvested LAB composition obtained following the implementation of the culturing and harvesting methods described herein, which has further undergone washing and/or freezing and/or drying and/or formulating.

- 25 By “*intermediate starter culture*”, as used herein it is meant one harvested LAB composition obtained by a culturing method, followed by a harvesting step, which has further undergone washing and/or freezing and/or drying and/or formulating.

- 30 The terms “*Intermediate starter culture*” and “*intermediate starter culture of the invention*” do not have an identical meaning to “starter culture” as defined below. One or more intermediate starter cultures may be mixed to form a starter culture, as discussed below.

In a particular embodiment, said intermediate starter culture of the invention has a concentration of LAB which is between 10^7 to 10^{12} or 10^8 to 10^{13} CFU/g, and more

preferably at least at least 10^7 , at least 10^8 , at least 10^9 , at least 10^{10} , at least 10^{11} or at least 10^{12} CFU/g of the intermediate starter culture.

In one embodiment, the LAB intermediate starter culture of the invention may be produced, obtained or obtainable from any harvested LAB or harvested LAB composition of the invention. In a particular embodiment, the intermediate starter culture of the invention is obtained from the harvested LAB or harvested LAB composition, after washing the LAB from a harvested LAB composition of the invention, and/or freezing and/or drying a harvested LAB composition of the invention and/or formulating a harvested LAB composition of the invention.

The term "washing" as used herein refers to the use of water or other liquid to remove any components from the LAB composition or harvested LAB composition of the invention. In one embodiment the harvested LAB composition is washed to form an intermediate starter culture. Suitably only minimal LAB are removed from the harvested LAB composition during washing.

In a particular embodiment, the intermediate starter culture of the invention is obtained from the harvested LAB or harvested LAB composition as described above, by freezing and/or drying a harvested LAB or harvested LAB composition of the invention.

In one embodiment, the LAB intermediate starter culture of the invention may be, liquid, solid, frozen, dried, freeze dried, in the form of pellets or frozen pellets, or in a powder or dried powder. In a particular embodiment, the intermediate starter culture of the invention is frozen, dried, freeze-dried, in the form of pellets or frozen pellets, or in a powder or dried powder.

In one embodiment, the LAB, the harvested LAB, the harvested LAB composition or LAB concentrate, as obtained in any embodiments defined above, in particular the LAB concentrate, is frozen, dried (e.g. spray dried) or freeze-dried in particular to obtain the intermediate starter culture of the invention as defined herein, preferably to obtain intermediate starter culture of the invention as frozen LAB pellets or as a powder or dried LAB powder.

Advantageously, when a freezing step is carried out, cryoprotectant(s) and/or stabilizer(s) is/are mixed with the harvested LAB before freezing. Cryoprotectants or

stabilizers include, but are not limited to, glucose, lactose, raffinose, sucrose, trehalose, adonitol, starch maltodextrin, glycerol, mannitol sorbitol, polypropylene glycol, polyethylene glycol, ribitol alginate, bovine serum albumin, carnitine, citrate, cystein, dextran, dimethyl sufoxide, sodium glutamate, glycin, betaine, glycogen, hypotaurin, skimmed milk peptone, polyvinyl pirrolidine, taurine, nucleosides, nucleotides and any combination thereof.

The LAB in the frozen, dried, or freeze dried intermediate starter cultures may be in a concentration of at least 10^8 , at least 10^9 , at least 10^{10} , at least 10^{11} or at least 10^{12} CFU/g of the intermediate starter culture.

In one embodiment, the intermediate starter culture of the present invention comprises between about 0.1% and 20% of remaining algae or a fraction thereof, calculated as weight by weight of the intermediate starter culture, when this intermediate starter culture is frozen. Thus, the invention is also directed to a frozen intermediate starter culture as defined herein, comprising between about 0.1% and 20% of remaining algae or a fraction thereof, calculated as weight by weight of the intermediate frozen starter culture.

Suitably, the frozen intermediate starter culture of the present invention comprises between about 0.5% and 15% of remaining algae or a fraction thereof, calculated as weight by weight of the frozen intermediate starter culture.

Suitably, the frozen intermediate starter culture of the present invention comprises between about 0.5% and 10% of remaining algae or a fraction thereof, calculated as weight by weight of the frozen intermediate starter culture

Preferably remaining algae (or a fraction thereof) may mean remaining spirulina or remaining chlorella.

The term "remaining algae" as used herein means the algae or a fraction thereof that was present during the culturing step and which remains in the LAB composition or the harvested LAB composition or the intermediate starter culture or the frozen intermediate starter culture.

In a particular embodiment, the invention disclosed herein also includes a dried intermediate starter culture obtained following drying of the frozen intermediate starter culture as defined herein, in particular following drying of the frozen intermediate starter culture of the present invention comprises between about 0.1% and 20% of remaining
5 algae or a fraction thereof, calculated as weight by weight of the intermediate starter culture.

In a particular embodiment, said LAB intermediate starter culture is in the form of frozen pellets or a dried powder. In a particular embodiment, said LAB intermediate starter
10 culture, preferably obtained or obtainable by the method above, has a concentration of LAB of at least 10^7 CFU/g of the intermediate starter culture, in particular of at least 10^8 , at least 10^9 , at least 10^{10} , at least 10^{11} , or at least 10^{12} CFU/g of the intermediate starter culture, and comprises remaining algae (or a fraction thereof), in particular remaining spirulina, remaining chlorella or a fraction thereof, and is optionally of green colour. In a
15 particular embodiment, said LAB intermediate starter culture comprises remaining spirulina or a fraction thereof and has a green colour.

In a particular embodiment, the intermediate starter culture, in particular in the form of pellets, has a colour which is not white to beige colour. Thus, in a particular embodiment,
20 the intermediate starter culture, in particular in the form of pellets, has a similar colour as the pigments of the algae or the fraction thereof. In the particular case of cyanobacteria, such as spirulina, or green algae, the intermediate starter culture, in particular in the form of pellets, has a green colour. In the particular case of red algae, the intermediate starter culture, in particular in the form of pellets, has a red colour.

25 In another embodiment, the intermediate starter culture, in particular in the form of pellets, has a white to beige colour. Any method (such as washing or enzymatic treatment) to remove partially or totally the pigments contained in the algae (or a fraction thereof) or the extract may be used in order to obtain a intermediate starter culture with a
30 white to beige colour.

These methods of pigment removal may be implemented at any stage of the method to produce a intermediate starter culture, in particular before adding the algae or the

fraction thereof with the LAB composition, or after harvesting the LAB composition to obtain a harvested LAB composition or an intermediate starter culture.

Starter cultures

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The invention also includes LAB starter cultures, methods of producing said starter cultures and uses of said starter culture.

10

By "*starter culture*", as used herein it is meant a mixture comprising or consisting of at least one intermediate starter culture of the invention as defined herein with at least one other intermediate starter culture (The other intermediate starter culture may be any intermediate starter culture, which is an intermediate starter culture of the invention or not). Each intermediate starter culture is produced by culturing separately, wherein at least one of the intermediate starter cultures is produced by the methods of the invention described herein.

15

In a particular embodiment, the starter culture comprises or consists of a mixture of one intermediate starter culture of the invention and one other intermediate starter culture.

20

In a particular embodiment, the starter culture comprises or consists of a mixture of one intermediate starter culture of the invention and two other intermediate starter cultures.

25

In a particular embodiment, the starter culture is a mixture comprising or consisting of two intermediate starter cultures of the invention. These two intermediate starter cultures of the invention are different from each other, in that they are cultured separately from each other using the methods of the invention.

30

In a particular embodiment, the starter culture of the invention comprises or consists of a mixture of one intermediate starter culture of the invention and two or more other intermediate starter cultures. In a particular embodiment, the starter culture is a mixture comprising two or more different intermediate starter cultures of the invention.

In one embodiment the intermediate starter cultures mixed to form the starter culture of the invention comprise different species, and/or different strains, and/or different combinations of species and strains of LAB.

- 5 In one embodiment the intermediate starter cultures mixed to form the starter culture of the invention comprise at least one different species, and/or at least one different strain, and/or at least one different combination of species and strains of LAB.

- 10 In another embodiment the intermediate starter cultures mixed to form the starter culture of the invention each comprise the same species, and/or strains, and/or combinations of species and strains of LAB.

- 15 In another embodiment the intermediate starter cultures mixed to form the starter culture of the invention each comprise at least one of the same species, and/or least one of the same strains, and/or least one of the same combinations of species and strains of LAB.

- 20 The starter culture of the invention may be frozen, dried, freeze dried, liquid, solid, in the form of pellets or frozen pellets, or in a powder or dried powder. In a particular embodiment, the starter culture of the invention is frozen, dried, freeze-dried, in the form of pellets or frozen pellets, or in a powder or dried powder.

- 25 A starter culture according to the invention is obtained by mixing several (at least 2, preferably 2 or 3) intermediate starter cultures, at least one being an intermediate starter culture of the invention. The invention also includes a method to manufacture a starter culture comprising mixing at least one intermediate starter culture of the invention and at least one intermediate starter culture. In a particular embodiment, the at least one intermediate starter culture of the invention and at least one intermediate starter culture are mixed under frozen form, in the form of pellets or frozen pellets. In a particular embodiment, the at least one intermediate starter culture of the invention and at least one intermediate starter culture are mixed under dried form, freeze-dried form, in the form of a powder or dried powder.
- 30

Use of intermediate starter culture or starter culture

The invention also provides the use of an intermediate starter culture or starter culture as defined above or as obtained or obtainable by any of the method as defined herein,

5 The intermediate starter culture or starter culture of the invention may preferably be used for increasing the acidity of a fermentable substrate and/or for producing a fermented product. Adding a intermediate starter culture or starter culture to a fermentable substrate (such as a fermentable medium) may also be referred to as seeding a substrate or medium, or seeding a substrate or medium to be fermented.

10 The invention also concerns the use of an intermediate starter culture or starter culture of the invention for preparing products, in particular food products or feed products, in particular fermented products, in particular fermented food products or fermented feed products.

15 The invention also provides a method for preparing a product, preferably a food or feed product, in particular fermented products, in particular fermented food products or fermented feed products, comprising a) seeding a medium with an intermediate starter culture or starter culture of the invention, b) optionally fermenting said medium, and c) obtaining said product. In a particular embodiment, said medium is milk, in particular milk
20 of animal origin. In a particular embodiment, said medium is milk, in particular milk of animal origin, and the fermented product is a dairy product, preferably a yoghurt, a cheese (such as an acid curd cheese, a hard cheese, a semi-hard cheese, a cottage cheese), a butter, a buttermilk, quark, a sour cream, kefir, a fermented whey-based beverage, a koumiss, a milk beverage, a yoghurt drink, a fermented milk, a matured
25 cream, a fromage frais, a milk, a dairy product retentate, a processed cheese, a cottage cheese, a cream dessert, or infant milk. The invention also concerns said product, in particular dairy products obtained by said preparation method.

30 The term "product" as used herein may also include such as meat products (such as salami or sausages), animal feed and pet food; surface coating material (such as paint), agricultural products (such as crops and seeds), feed bakery, wine, beer or other beverages, or vegetable products and pharmaceutical industry products. Furthermore, the intermediate starter cultures or starter cultures of the invention may also be used to produce proteins including enzymes and various kinds of useful compounds.

A “food” or “feed” as used herein may be an animal or human food or feed.

Formulating

- 5 The LAB composition, harvested LAB composition, intermediate starter cultures and/or starter culture of the present invention may be formulated into any suitable form.

Formulating may include pelleting, capsules, caplets, tableting, blending, coating, layering, formation into chewable or dissolvable tablets, formulating into dosage
10 controlled packets, formulating into stick packs and powdering.

Formulating may also include the addition of other ingredients to the LAB composition, harvested LAB composition, intermediate starter cultures and/or starter culture of the present invention. Suitable ingredients include for example food ingredients, sugars,
15 carbohydrates, and dairy products.

In one embodiment formulating does not include the addition of any further LAB.

Packaging

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The LAB composition, harvested LAB composition, intermediate starter culture and/or starter culture of the present invention may be packaged.

In one embodiment, packaging occurs after harvesting the LAB composition. In one
25 embodiment, packaging occurs after freezing the harvested LAB composition. In one embodiment, packaging occurs after drying the harvested LAB composition. In one embodiment, packaging occurs after mixing intermediate starter culture(s).

Suitably the packaging may be comprised of a vacuum pack, sachet, box, a blister pack,
30 stick pack, or tin.

Probiotics

The LAB composition or harvested LAB composition of the invention may be used as, or as components of, probiotics and/or prebiotics.

Here, a prebiotic is:

5 *"a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of beneficial bacteria".*

The term "probiotic" as used herein defines live microorganisms (including bacteria or yeasts for example) which, when for example ingested or locally applied in sufficient numbers, beneficially affects the host organism, i.e. by conferring one or more demonstrable health benefits on the host organism. Probiotics may improve the microbial balance in one or more mucosal surfaces. For example, the mucosal surface may be the intestine, the urinary tract, the respiratory tract or the skin. The term "probiotic" as used herein also encompasses live microorganisms that can stimulate the beneficial branches of the immune system and at the same time decrease the inflammatory reactions in a mucosal surface, for example the gut.

Whilst there are no lower or upper limits for probiotic intake, it has been suggested that at least 10^6 - 10^{12} , preferably at least 10^6 - 10^{10} , preferably 10^8 - 10^9 CFU/ml as a daily dose will be effective to achieve the beneficial health effects in a subject.

The LAB composition and the harvested LAB composition of the invention may be used a probiotics or prebiotics. The LAB composition or harvested LAB composition may be added to other components to create composition for use as probiotics and/or prebiotics. They may be used a direct fed microbials in food or feed for humans or animals (such as cattle). The probiotics and/or prebiotics may be used as a food or feed additive for humans or animals (such as cattle).

25 The invention also provides a method for preparing a probiotic or prebiotic product, preferably a food or feed product for humans or animals. This method comprises creating a LAB composition of the invention, or harvested LAB composition of the invention, and using this as a probiotic or prebiotic, either directly or in a composition.

30 The probiotics and prebiotics of the invention may be frozen, dried, freeze dried, liquid, solid, in the form of pellets or frozen pellets, or a in powder or dried powder.

The invention may also be described by the following numbered paragraphs:-

1. A method of producing a lactic acid bacteria composition with increased lactic acid
5 bacteria viable active biomass, comprising the steps of
- (i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;
- (ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce said LAB composition;
- 10 wherein said increased viable active biomass is defined as at least one of the following:
- a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae; and/or
- 15 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae.
- 20 2. Use of an algae or a fraction thereof, in the manufacture of a lactic acid bacteria composition with increased viable active biomass of lactic acid bacteria, cultured under aerobic growth conditions, wherein increased viable active biomass is defined as at least one of the following:
- a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid
25 bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae; and/or
- b) wherein the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the

content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae.

3. The method according to paragraph 1 or the use according to paragraph 2 wherein in particular said algae is a prokaryotic algae, such as a cyanobacteria, or an eukaryotic algae selected from the group consisting of a red algae and a green algae, or any fraction thereof, and more particularly wherein said algae is selected from the group consisting of spirulina, chlorella and a fraction thereof.

4. The method or use according to any preceding numbered paragraph, wherein the Ta value of a lactic acid bacteria composition obtained after culture in the presence of said algae under aerobic conditions, is at least 12, at least 15, at least 20, at least 30 or at least 40 minutes less than the Ta value of a lactic acid bacteria culture medium obtained after culture in the absence of said algae.

5. The method or use according to any preceding numbered paragraph, wherein the content of viable cells of a lactic acid bacteria composition obtained after culture in the presence of said algae under aerobic conditions, is at least 130, at least 140, at least 150 or at least 200 % the content of viable cells of a lactic acid bacteria culture medium obtained after culture in the absence of said algae.

6. The method or use according to any preceding numbered paragraph, wherein further the optical density (OD) gain of the lactic acid bacteria composition is of at least 120, at least 130, at least 140, at least 150, at least 200 or at least 300 % the OD gain of a lactic acid bacteria culture medium or a same sized sample thereof obtained after culture in the absence of said algae.

7. The method or use of any preceding numbered paragraph wherein:

(i) the Ta value is determined by Assay I as presented herein; and/or

(ii) the CFU is determined by Assay II as presented herein; and/or

(iii) the OD gain is determined by Assay III as presented herein.

8. The method or use according to any preceding numbered paragraph wherein said algae is used or is present or is added into said culture medium, at a concentration of at least 0.1 g/L, at least 0.5 g/L, in particular at least 1g/L of culture medium.

5 9. The method or use according to any preceding numbered paragraph wherein said lactic acid bacteria composition consists of a strain from species *Lactococcus* or a strain from species *Leuconostoc*, or comprises at least a strain from species *Lactococcus* or at least a strain from species *Leuconostoc*.

10 10. The method or use according to any preceding numbered paragraph wherein, in calculating the Ta value, the lag phase of growth is carried out on a fermentable substrate which is milk.

11. A method or use according to any of numbered paragraphs 1 to 10 wherein lactic acid bacteria are harvested.

15 12. A lactic acid bacteria starter culture obtained by the method or use of any one of numbered paragraphs 1-11.

13. A lactic acid bacteria starter culture according to numbered paragraph 12, which comprises at least 0.1% of remaining algae or a fraction thereof, calculated as weight by weight of the starter culture.

20 14. A lactic acid bacteria starter culture according to any of numbered paragraphs 12 to 13, which is frozen, dried, freeze dried, liquid, solid, in the form of pellets or frozen pellets, or a powder or dried powder.

15: Use of a starter culture according to any of numbered paragraphs 12 or 14, for increasing the acidity of a fermentable substrate and/or for producing a fermented product. .

25 16. A method of producing a harvested lactic acid bacteria composition, comprising the steps of

(i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

(ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

5 a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

10 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

15 (iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed to produce a harvested LAB composition.

17. A method of producing a harvested lactic acid bacteria composition, comprising the steps of

20 (i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

(ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

25 a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

- 5 (iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed, to produce a harvested LAB composition.

18. A method of producing a harvested lactic acid bacteria composition, comprising the steps of

- 10 (i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

(ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at
15 least one of the following:

a) the lag phase of growth on a fermentable substrate (T_a value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the T_a value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

- 20 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

(iii) harvesting the LAB from the LAB composition of step ii).

- 25 19. A method of producing a harvested lactic acid bacteria composition having increased lactic acid bacteria viable active biomass, comprising the steps of

(i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

(ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

5 a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

10 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

15 (iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed, to produce a harvested LAB composition.

20. A method of producing a harvested lactic acid bacteria composition having increased lactic acid bacteria viable active biomass, comprising the steps of

(i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

20 (ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

25 a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content

of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

- (iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed to produce a harvested LAB composition.

EXAMPLES

I. Material and methods

1.1. Strains

Three lactic acid bacteria strains have been studied: the *Lactococcus lactis* DGCC2631, the *Lactococcus lactis* DGCC9209 and the *Leuconostoc mesenteroides* DGCC4634.

The *Lactococcus lactis* DGCC2631 has been deposited under the Budapest Treaty on September 24, 2013 in the name of Danisco Deutschland GmbH at the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH (Inhoffenstr. 7B, D-38124 Braunschweig), under number DSM 27777. The depositor (Danisco Deutschland GmbH) authorises the applicant to refer to the deposited biological material in the application, and gives unreserved and irrevocable consent to the deposited material being made available to the public.

1.2. Algae and hemin

The following algae have been assayed, at a concentration of 1, 2 and 5g/L of growth medium:

Algae	Provider	Reference / lot
Spirulina*	Setalg	F1280611N
Spirulina*	SEAH (Lot 1)	069094
Spirulina*	SEAH (Lot 2)	A110047612
Spirulina*	COPCI	65%_2829 (NBC 20120928)
Spirulina*	AlphaBiotec Miette	

Chlorella (<i>Chlorella vulgaris</i>)	Setalg	A416 0811N
Laminaria	Setalg	A20090810G
Fucus	Setalg	A20270912N

*: *Arthrospira platensis*

A stock solution of hemin at 0.05 % is prepared by dissolving 100 mg of hemin (Sigma, product number 51280, porcine origin) in 2 ml of 5N NaOH, to which 198 ml of water is then added; the solution is autoclaved at 120°C for 20 minutes.

1.3. Culture medium A

The following media is used as the culture medium for all LAB in the following examples:

10

Component	Supplier	Unit	medium A (control)	medium A with algae	medium A with hemin
Lactose	BMI	g	47.00	47.00	47.00
Yeast extract 0207 / MG-L (powder)	Springer	g	21.00	21.00	21.00
MgSO ₄ 7 H ₂ O	Univar	g	0.63	0.63	0.63
MnSO ₄ , H ₂ O	SVPC	g	0.15	0.15	0.15
Na ascorbate	Univar	g	0.90	0.90	0.90
Defoamer DC1520	Dow corning	g	0.01	0.01	0.01
Algae powder	/	g	/	1 to 5	/
Soft water, qsp	/		1000	1000	1000
			Sterilisation 20 min at 121 °C		
Hemin (0.05% stock solution)	/	mL	/	/	20

Medium A (control) is the media used in the LAB culture medium (control).

1.4. Culture conditions

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In a 1L Erlenmeyer flask, 100 ml of the above media (either with algae, or hemin or as a control) is inoculated with a selected lactic acid bacteria, at a rate of 0.01%

(corresponding to an inoculation of 1.10^7 to 3.10^7 cfu/ml of media). The inoculated media is then incubated for 18h at 30°C on a shaker at 160 rpm (INFORS shaker).

1.5. Ta measurement

5

Each cultured LAB is tested to qualify its ability to acidify milk. The milk substrate used is prepared by dissolving 90gr of skim milk powder from Humana into deionised water at a 9/100 dilution (for example 90gr skim milk powder into 910gr of water. Rehydration takes place for 30mn at room temperature under stirring conditions and is followed by

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sterilising the reconstituted milk in 480ml volume at 96°C for 30min. Milk activity tests are pre warmed at 30°C and then inoculated at 0.1% weight by weight with the LAB composition of the invention. pH is recorded for each milk test, for 16h at 30°C, using CINAC equipment from Alliance Instruments which is 32 channel pH probe recorder. The time (minutes) to decrease the pH by 0.08 pH units is calculated for each sample.

15

Comparison is made to a LAB composition which is identical except it is cultured without algae (the LAB culture medium (control)), or hemin culture (control plus hemin, as described in 1.3 above). In this experiment the LAB is not treated in any way after culture before it is used to measure the Ta. However, if the culturing is carried out in a bioreactor, or any liquid form, separation of the LAB, such as by centrifugation, may be

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necessary before the Ta measurement may be carried out.

1.6. CFU measurement

1 ml of LAB composition is diluted by mixing in 9ml of TS tryptone salt diluent at room temperature to obtain a dilution 10⁻¹ after mixing on a vortex for 30s; this dilution procedure is repeated by inoculation of 1 ml of dilution 10⁻¹ into 9 ml of TS diluent till 10⁻⁸. Proper inoculums of dilutions 10⁻⁷ and 10⁻⁸ are then plated on M17 agar using Eddy jet spiral inoculator's and incubated under anaerobic conditions at 30°C for 48h. At the end of incubation, grown colonies are enumerated and CFU (colony forming unit) final result determined after multiplication by the dilution factor.

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The CFU of the LAB culture medium (control cultured under the same aerobic conditions) and hemin culture are also measured in the same way.

1.7. OD measurement

1ml of LAB composition is diluted into 9ml, or in 18 or in 27 ml of distilled water in order to obtain a diluted sample (and an OD range comprised between 0.1 and 0.5 on diluted sample). The diluted sample is then measured at 660nm in a Thermo Scientific Genesys 20™ spectrophotometer (with a spectral slit width specification of 8nm and effective optical path of 10mm); the OD (Optical Density) is calculated by multiplying the dilution factor of the bacterial culture by the value given by the spectrophotometer. Calculated OD is the multiplication of the dilution factor by the value given by the spectrophotometer.

The OD of the LAB culture medium (control cultured under the same aerobic conditions) and hemin culture are also measured in the same way.

II. Results

Results are provided in Figures 1 to 6, and Tables 1 to 11. For each sample, Tables 1 to 3 and 5 to 11 describe, from the left to the right: the nature of the cultured LAB, the nature of the added algae [hemin means that hemin is added instead of algae; control means that no compound (no algae, no hemin) is added. i.e. this is the LAB culture medium], the supplier (if needed), the concentration (if needed), and the 6 following parameters:

- (i) delta OD (or OD gain), which corresponds to the difference between the OD of the cultured LAB at the end of the culture under aerobic conditions minus the OD of the same contents (LAB in media plus algae or hemin or neither as listed in the table) before culture;
- (ii) Ta, which corresponds to the time (in minutes) necessary for the cultured LAB obtained after culture under aerobic conditions, to decrease the pH of the milk by 0.08 pH units, as per the above;
- (iii) CFU, which corresponds to the colony-forming units obtained for the cultured LAB obtained after the culture under aerobic conditions, as described above;

- (iv) ratio delta OD with algae over delta OD control (in %), which corresponds to the ratio of (1) the delta OD of a LAB composition of the invention (cultured under aerobic conditions in the presence of the algae) over (2) the delta OD of a control (the LAB culture medium as defined above - LAB cultured in the same conditions, same LAB, same growth conditions). For tables 7 to 11, when sample is hemin, the ratio corresponds to the ratio of the delta OD of a culture grown under aerobic conditions in the presence of hemin over the delta OD of a control culture grown in the same conditions. The control delta OD used for these calculations is the one described in the same Table. A percentage above 120% means that addition of the algae has a significant effect on the OD increase;
- (v) difference between the Ta of the composition of the invention (Ta with algae) minus the Ta of the LAB culture medium (Ta control) in minutes. For tables 7 to 11, when sample is hemin, the difference corresponds to the difference between the Ta of a LAB composition after culture under aerobic conditions in the presence of hemin minus the Ta of a control LAB after culture in the same conditions. The control Ta used for these calculations is the one described in the same Table. A difference more than 10 minutes means that addition of the algae has a significant effect on Ta reduction. This means at least 5%, at least 10%, at least 15%, at least 20% difference compared to the control.
- (vi) ratio CFU with algae over CFU control (in %), which corresponds to the ratio of the CFU of a the LAB composition of the invention over the CFU of the LAB culture medium (control) composition For tables 7 to 11, when sample is hemin, the ratio corresponds to the ratio of the CFU of a LAB composition after culture under aerobic conditions in the presence of hemin over the CFU of a control culture grown in the same conditions. The control CFU used for these calculations is the one described in the same Table. A percentage above 120% means that the addition of the algae has a significant effect on the CFU increase (20% more than the control).

Furthermore, for comparison the Ta and CFU values for the same cultures but under anaerobic culture conditions are also given in Tables 1-11. For convenience only 2 or 3 significant figures appear to each column of the Tables (1-16) showing the experimental results. However calculations, such as calculating ratio CFU with algae over CFU control (in %), were carried out using the entire values of 3 or more significant figures. For example the values shown in column 10 of table 1 were calculated using the entire, 3 significant figures or more, results of column 7, even though only two significant figures appear in each row of column 7.

2.1. Identification of the growth effect of spirulina on LAB biomass and activity under aerobic conditions

3 different spirulina extracts, from 2 different suppliers, at 3 different concentrations (1, 2 and 5 g/L) have been tested in a culture of *Lactococcus lactis*. As shown in Table 1:

- addition of spirulina at 1 to 5gr/L increases the OD gain between 211 and 262% as compared to a control culture, confirming that spirulina increases the biomass of *Lactococcus lactis*.

- addition of spirulina at 1 to 5gr/L increases the CFU, between 155 and 280% as compared to a control culture, confirming that the increased biomass of *Lactococcus lactis* following spirulina addition is a viable biomass.

Interestingly, as compared to a control culture (LAB culture medium), the Ta in milk of a LAB composition of the invention, cultured with spirulina, is reduced between 28 and 56 minutes, confirming that the addition of spirulina not only increases the biomass of viable *Lactococcus lactis*, but also increases the biomass of viable active *Lactococcus lactis*.

The results of the SEAH lot 1 and control culture are also shown on Figure 1 (Ta), Figure 3 (CFU) and Figure 5 (OD).

The first experiment has been confirmed using spirulina from 2 different suppliers. Results are presented in Table 2. SEAH2 (or SEAH Lot 2 a used in Section 1.2 above) is the same spirulina as the one used in Table 1. Thus, addition of spirulina at 1 to 5gr/L

increases the OD gain of the LAB composition of the invention between 228 and 240% as compared to a control culture, increases the CFU between 220 and 229% as compared to a control culture, and reduces the Ta between 28 and 44 minutes as compared to a control culture. This confirms the effect of spirulina addition under aerobic growth conditions on the increase of the viable active biomass of *Lactococcus lactis*.

The control culture is the LAB culture medium as previous defined.

2.2. Assays on further algae: identification of the growth effect of Chlorella on LAB biomass and activity under aerobic conditions

Spirulina is a blue-green algae (cyanobacteria). Further assays have been done on the same *Lactococcus lactis* strain, with 3 different algae: one green algae (Chlorella) and 2 brown algae (Laminaria and Fucus). SEAH2 is the same spirulina as the one used in Table 1.

Results are disclosed in Table 3:

- addition of chlorella at 1 to 5gr/L increases the OD gain between 159 and 373% as compared to a control culture, confirming that chlorella increases the biomass of *Lactococcus lactis*.

- addition of chlorella at 1 to 5gr/L give similar results (96%) or slightly increases the CFU (between 132 and 151%) as compared to a control culture, confirming that the increased biomass of *Lactococcus lactis* following spirulina addition is a viable biomass.

Interestingly, as compared to a control culture, the Ta of a LAB composition of the invention cultured with chlorella addition is reduced between 20 and 28 minutes, confirming that the addition of chlorella not only increases the biomass of viable *Lactococcus lactis*, but also increases the biomass of viable active *Lactococcus lactis*.

The results of the effect of chlorella on the cultured LAB (the composition of the invention) compared to control culture are also shown on Figure 2 (Ta), Figure 4 (CFU)

and Figure 6 (OD). The control culture is the LAB culture medium as previous defined above.

Interestingly and in contrast to what is observed with spirulina and chlorella, the addition of either Laminaria or Fucus does not increase the OD gain of a *Lactococcus lactis* culture under aerobic growth (99-107%). Moreover, the CFU of a LAB composition cultured with either Laminaria or Fucus under aerobic growth reaches only between 72% and 94% the CFU on a control culture. Finally, the Ta of a LAB composition cultured with either Laminaria or Fucus under aerobic growth is similar (0 min) or increases (up to 12 minutes) as compared to a control culture.

Consequently, these results show that not all the algae can be used to increase the biomass of lactic acid bacteria under aerobic growth conditions. In particular, brown algae, such as Laminaria or Fucus, give worse results than a control culture. In contrast, inventors have interestingly shown that both spirulina (cyanobacteria, also known as blue-green algae) and chlorella (green algae) are able to significantly increase not only the biomass of *Lactococcus lactis*, but also the active biomass of *Lactococcus lactis*.

2.3. The effect of algae without LAB strain

In order to check that the OD gain increase is not due to algae degradation during aerobic growth, and that the Ta reduction in milk is not due to the booster effect of the algae (spirulina or chlorella), the same assay as above, but without LAB inoculation has been performed. No CFU value was calculated.

Table 4 clearly shows that, in absence of LAB, the ratio of OD gain is reduced as compared to a control (a LAB culture medium as previous defined).

Moreover, in absence of LAB, the Ta is increased from at least 300 minutes (up to 648 minutes) as compared to a control. The same type of results has been obtained with both spirulina and chlorella.

These results confirm that the effect of the spirulina or chlorella on the OD gain increase, CFU increase and on the Ta reduction is due to the effect of spirulina or chlorella on the growth of active LAB under aerobic conditions.

5 **2.4. Confirmation of the spirulina growth effect in further lactic acid bacteria**

The effect of spirulina has been assayed on two other lactic acid bacteria composition: a further *Lactococcus lactis* and a *Leuconostoc mesenteroides*. Results are disclosed in Tables 5 and 6, respectively.

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Regarding the other *Lactococcus lactis* strains, the inventors have found:

- addition of spirulina at 1 to 5gr/L increases the OD gain between 156 and 279%, as compared to a control.

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- addition of spirulina at 1 to 5gr/L decreases the CFU (64%) at a 1g/L concentration, give similar results of CFU (97%) at a 2g/L concentration or slightly increases the CFU (127%) at a 5g/L concentration, as compared to a control, and

20

- the Ta of a LAB composition of the invention cultured with spirulina addition is reduced between 24 and 60 minutes, as compared to a control.

The control culture is the LAB culture medium as previous defined.

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This confirms that spirulina has a clear effect at least on OD gain increase and on Ta reduction, regarding culture of *Lactococcus lactis* strains under aerobic conditions.

Regarding the *Leuconostoc mesenteroides* strain:

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- addition of spirulina at 1 to 5gr/L increases the OD gain between 107 and 183%, as compared to a control;

- addition of spirulina at 1 to 5gr/L increases the CFU, between 123 and 145%, as compared to a control, and

- the Ta of a LAB composition of the invention cultured with spirulina addition is reduced between 12 and 16 minutes, as compared to a control.

The control culture is the LAB culture medium as previous defined.

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This confirms that spirulina has also an effect on OD gain increase, on CFU increase and on Ta reduction, regarding culture of a *Leuconostoc mesenteroides* strain under aerobic conditions.

10 Altogether, these results show that addition of green algae (such as chlorella) and cyanobacteria (such as spirulina) is able to increase, under aerobic growth conditions, the active biomass of lactic acid bacteria, at least *Lactococcus* species, such as *Lactococcus lactis*, and *Leuconostoc* species, such as *Leuconostoc mesenteroides*:

15 2.5. Comparison of the spirulina effect with hemin effect

The effect of the addition of spirulina and/or chlorella on the culture under aerobic growth conditions of *Lactococcus lactis* and *Leuconostoc mesenteroides* has been compared to the addition of hemin. The results are reported in Tables 7 to 11.

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Tables 7 and 8 show that the addition of spirulina gives better results in terms of Ta reduction (36 versus 52 minutes) as compared to the addition of hemin, with the first *Lactococcus lactis* strain DBCC2631. Table 9 shows that for the other *Lactococcus lactis*, the addition of spirulina gives similar results as hemin addition (60-minute Ta reduction). Table 10 shows that the addition of chlorella gives similar results as hemin addition (-28 minutes versus -32 minutes for Ta measurement). Finally, regarding the *Leuconostoc mesenteroides*, the addition of spirulina gives better results in terms of Ta reduction as compared to the addition of hemin (hemin increases the Ta as compared to the control).

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Consequently, it has been shown that spirulina (a cyanobacteria) and/or chlorella (a green algae) are able to increase not only the active biomass of lactic acid bacteria (such as *Lactococcus* or *Leuconostoc*) when cultured under aerobic growth conditions, but also at a level which is at least similar to the level obtained with hemin addition.

2.6. Comparative of Aerobic and Anaerobic Results

For comparison purposes, cultures under anaerobic growth conditions were also carried out. The same growth conditions apply (lactic acid bacteria strains, media type, inoculation rate, temperature, culture time etc), except that tests were run under static conditions [absence of oxygen].

The results are presented separately in Tables 12 to 16, which describe, from the left to the right: the nature of the cultured LAB, the nature of the added algae [hemin means that hemin is added instead of algae; control means that no compound (no algae, no hemin) is added. i.e. this is the LAB culture medium], the supplier (if needed), the concentration (if needed), and the 6 following parameters:

- (i) Ta aerobic, which corresponds to the time (in minutes) necessary for the cultured LAB obtained after culture under aerobic conditions, to decrease the pH of the milk by 0.08 pH units, as per the above (e.g. as per Assay I);
- (ii) CFU aerobic, which corresponds to the colony-forming units obtained for the cultured LAB obtained after the culture under aerobic conditions, as described above (e.g. as per Assay II);
- (iii) Ta anaerobic, which corresponds to the time (in minutes) necessary for the cultured LAB obtained after culture under anaerobic conditions, to decrease the pH of the milk by 0.08 pH units, as per the above (e.g. as per Assay I);
- (iv) CFU anaerobic, which corresponds to the colony-forming units obtained for the cultured LAB obtained after the culture under anaerobic conditions, as described above (e.g. as per Assay II);
- (v) The difference between the Ta aerobic and Ta anaerobic value of the culture, which corresponds to (i) minus (iii).

- (vi) The ratio CFU aerobic over CFU anaerobic (in %), which corresponds to the percentage change of (ii) compared to (iv) (calculated as (ii) divided by (iv) and then multiplied by 100).

5 As per the above, for convenience only 2-3 significant figures are shown for each value in Tables 12-16. However, calculations were carried out using 3 significant figures or more.

Gain of Ta between LAB cultured with algae under aerobic growth conditions and LAB cultured with algae under anaerobic algae

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Tables 12 to 16 illustrate the Ta difference between LAB cultured under aerobic and anaerobic growth conditions.

In absence of algae during the culture (control), the Ta difference is only the result of aeration [oxygen addition], and is comprised between -16 and +20 min. Thus, for DGCC9209, aerobic conditions have no impact on Ta (difference is 0) (Table 15). For DGCC4634, aerobic conditions increase the Ta by 20 minutes (Table 16). For DGCC2631, the aerobic conditions decrease the Ta, even in absence of algae, but only between 4 and 16 minutes (Tables 12 to 14).

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The use of algae in addition to aerobic growth conditions drastically decreases the Ta, as compared to culture with algae but in anaerobic conditions. Thus, for DGCC2631, the decrease is up to -84 minutes (Table 13) as compared to anaerobic conditions, and is on average between 20 and 45 minutes (Tables 12 to 14). For DGCC9209, the Ta decrease is comprised between 24 and 52 minutes, knowing that the aerobic conditions have a negligible or little impact on Ta in the control (Table 14). Finally, for DGCC4634, whereas aerobic conditions increase the Ta, both in the control and in presence of hemin, the addition of algae decreases the Ta between 8 and 12 minutes (Table 16).

30 Interestingly, for all the assays, the values of delta Ta [delta Ta aerobic minus Ta anaerobic] were more negative (*i.e.*, the Ta was shorter) in presence of algae than in absence of algae, what confirms the beneficial effect of algae under aerobic conditions on Ta reduction. As compared to hemin, the values of delta Ta [delta Ta aerobic minus

Ta anaerobic] were identical or more negative (*i.e.*, the Ta was identical or shorter) using algae, confirming the effect of using algae instead of hemin.

CFU increase, between LAB cultured with algae under aerobic growth conditions and LAB cultured with algae under anaerobic algae

Tables 12 to 16 illustrate the ratio of the CFU of LAB cultured under aerobic growth conditions over the CFU of LAB cultured under anaerobic growth conditions.

In absence of algae during the culture (control), a ratio CFU aerobic over CFU anaerobic above 100% (meaning more viable LAB after culturing in aerobic conditions than after culturing in anaerobic conditions) is only the result of aeration, and was found to be between 105 and 123%. Aeration has a larger effect on DGCC9209 with an increase of the ratio CFU aerobic over CFU anaerobic of about 300% in the control (Table 15).

The use of algae in addition to aerobic growth conditions increases the ratio CFU aerobic over CFU anaerobic of at least 200% and up to 500% for DGCC2631. This increase is in the same range as hemin (tables 12 to 14). For DGCC9209 and DGCC4634, the increase of the ratio CFU aerobic over CFU anaerobic is similar to or a slightly higher than the control, and is between 235 and 325% for DGCC9209 (Table 15) and is about 140% for DGCC4634 (Table 16).

Altogether these results show that LAB culturing under aerobic conditions and in presence of algae (*spirulina* and *chlorella*) not only increases the biomass of viable cells, but also drastically increases the biomass of viable active cells, both when compared to control and hemin cultured under aerobic conditions and when compared to anaerobic cultures.

Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced without departing from

the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention which is delineated by the appended claims.

5 All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference. Documents incorporated by reference into this text should not be construed as an admission that such a document is "prior art" to the present application.

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC2631	Control	x	x	1.56	244	1.4	100	0	100
DGCC2631	Spirulina	Setalg	1	3.29	216	2.1	211	-28	155
			2	3.85	200	2.3	247	-44	167
			5	3.88	208	2.9	248	-36	207
DGCC2631	Spirulina	SEAH lot 1	1	3.74	220	2.6	240	-24	185
			2	4.03	212	3.1	258	-32	224
			5	4.09	192	3.9	262	-52	280
DGCC2631	Spirulina	SEAH lot 2	5	3.93	188	3.0	252	-56	220

Table 1

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC2631	Control	x	x	1.50	224	1.20	100	0	100
DGCC2631	Spirulina	COPCI	5	3.43	196	2.6	228	-28	220
DGCC2631	Spirulina	SEAH lot 2	5	4.00	188	2.7	266	-36	227
DGCC2631	Spirulina	AlphaBiotec	1	3.60	156	2.8	240	-68	229
			2	3.58	180	2.8	238	-44	229

Table 2

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC2631	Control	x	x	1.69	228	1.7	100	0	100
DGCC2631	Spirulina	SEAH lot 2	5	4.84	184	2.5	287	-44	149
DGCC2631	Chlorella	Setalg	1	2.69	208	1.6	159	-20	96
			2	4.01	200	2.6	237	-28	151
			5	6.30	200	2.3	373	-28	132
DGCC2631	Laminaria	Setalg	1	1.68	232	1.3	100	4	78
			2	1.70	228	1.3	101	0	74
			5	1.68	232	1.2	99	4	72
DGCC2631	Fucus	Setalg	1	1.69	232	1.4	100	4	81
			2	1.70	232	1.2	100	4	72
			5	1.81	240	1.6	107	12	94

Table 3

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC2631	Control	x	x	1.7	223	nd	100	0	nd
no strain	Spirulina	SEAH lot 1	1	-0.2	541	nd	-5	321	nd
			2	-0.1	524	nd	-3	312	nd
			5	0.1	507	nd	1	507	nd
DGCC2631	Control	x	x	2	232	nd	100	0	nd
no strain	Chlorella	Setalg	1	0.4	820	nd	14	612	nd
			2	1.1	848	nd	27	648	nd
			5	-0.6	480	nd	-10	280	nd

Table 4

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC9209	Control	x	x	1.4	236	2.5	100	0	100
DGCC9209	Spirulina	SEAH lot 2	1	2.2	212	1.6	156	-24	64
			2	3.4	184	2.4	237	-52	97
			5	4.0	176	3.1	279	-60	127

Table 5

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae / delta OD control (%)	Ta with algae minus Ta control	ratio CFU with algae / CFU control (%)
DGCC4634	Control	x	x	2.3	244	0.84	100	0	100
DGCC4634	Spirulina	SEAH lot 2	1	2.4	232	1.0	107	-12	123
			5	4.1	228	1.2	183	-16	145

Table 6

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae or with hemin / delta OD control (%)	Ta with algae or with hemin minus Ta control	ratio CFU with algae or with hemin / CFU control (%)
DGCC2631	Control	x	x	1.56	244	1.4	100	0	100
	hemin	Sigma	0.01	4.71	208	3.1	301	-36	225
	Spirulina	SEAH lot 1	5	4.09	192	3.9	262	-52	280

Table 7

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae or with hemin / delta OD control (%)	Ta with algae or with hemin minus Ta control	ratio CFU with algae or with hemin / CFU control (%)
DGCC2631	Control	x	x	1.50	224	1.20	100	0	100
	hemin	Sigma	0.01	4.58	202	3.11	305	-22	259
	Spirulina	AlphaBiotec	1	3.60	156	2.8	240	-68	229

Table 8

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae or with hemin / delta OD control (%)	Ta with algae or with hemin minus Ta control	ratio CFU with algae or with hemin / CFU control (%)
DGCC9209	Control	x	x	1.4	236	2.5	100	0	100
	hemin	Sigma	0.01	4.4	176	3.2	309	-60	128
	Spirulina	SEAH lot 2	5	4.0	176	3.1	279	-60	127

Table 9

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta aerobic (min)	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae or with hemin / delta OD control (%)	Ta with algae or with hemin minus Ta control	ratio CFU with algae or with hemin / CFU control (%)
DGCC2631	Control	x	x	1.69	228	1.7	100	0	100
	hemin	Sigma	0.01	4.43	196	2.8	262	-32	162
	Chlorella	Setalg	5	6.30	200	2.3	373	-28	132

Table 10

Strain	Sample name	Supplier	Concentration (g/l)	delta OD aerobic	Ta (min) aerobic	CFU/mL aerobic (10 ⁹)	ratio delta OD with algae or with hemin / delta OD control (%)	Ta with algae or with hemin minus Ta control	ratio CFU with algae or with hemin / CFU control (%)
DGCC4634	Control	x	x	2.3	244	0.84	100	0	100
	hemin	Sigma	0.01	2.5	256	0.86	110	12	101
	Spirulina	SEAH lot 2	5	4,1	228	1.2	183	-16	145

Table 11

Strain	Sample name	Supplier	Concentration (g/l)	Aerobic conditions		Anaerobic conditions		Delta Ta aerobic - Ta anaerobic (min)	Ratio CFU aerobic / CFU anaerobic (%)
				Ta (min)	CFU/mL (10 ⁹)	Ta (min)	CFU/mL (10 ⁹)		
DGCC2631	Control	x	x	244	1.4	248	1.1	-4	128
DGCC2631	hemin	Sigma	0,01	208	3.1	244	1.0	-36	310
DGCC2631	Spirulina	Setalg	1	216	2.1	236	0.98	-20	218
			2	200	2.3	236	1.1	-36	219
			5	208	2.9	228	1.1	-20	265
DGCC2631	Spirulina	SEAH lot 1	1	220	2.6	232	1.1	-12	243
			2	212	3.1	228	0.9	-16	342
			5	192	3.9	236	0.75	-44	513
DGCC2631	Spirulina	SEAH lot 2	5	188	3.0	232	1	-44	295

Table 12

Strain	Sample name	Supplier	Concentration (g/l)	Aerobic conditions		Anaerobic conditions		Delta Ta aerobic -Ta anaerobic (min)	Ratio CFU aerobic / CFU anaerobic (%)
				Ta (min)	CFU/mL (10 ⁹)	Ta (min)	CFU/mL (10 ⁹)		
DGCC2631	Control	x	x	224	1.20	240	1.14	-16	105
DGCC2631	hemin	Sigma	0,01	202	3.11	244	1.02	-42	305
DGCC2631	Spirulina	COPCI	5	196	2.6	240	1.1	-44	230
DGCC2631	Spirulina	SEAH lot 2	5	188	2.7	240	0.94	-52	290
DGCC2631	Spirulina	AlphaBiotec	1	156	2.8	240	0.92	-84	298
			2	180	2.8	232	0.99	-52	277

Table 13

Strain	Sample name	Supplier	Concentration (g/l)	Aerobic conditions		Anaerobic conditions		Delta Ta aerobic -Ta anaerobic (min)	Ratio CFU aerobic / CFU anaerobic (%)
				Ta (min)	CFU/mL (10 ⁹)	Ta (min)	CFU/mL (10 ⁹)		
DGCC2631	Control	x	x	228	1.7	232	1.5	-4	113
DGCC2631	hemin	Sigma	0,01	196	2.8	232	1.2	-36	239
DGCC2631	Spirulina	SEAH lot 2	5	184	2.5	228	0.8	-44	316
DGCC2631	Chlorella	Setalg	1	208	1.6	244	0.8	-36	203
			2	200	2.6	264	1.6	-64	161
			5	200	2.3	272	0.63	-72	360

Table 14

Strain	Sample name	Supplier	Concentration (g/l)	Aerobic conditions		Anaerobic conditions		Delta Ta aerobic -Ta anaerobic (min)	Ratio CFU aerobic / CFU anaerobic (%)
				Ta (min)	CFU/mL (10 ⁹)	Ta (min)	CFU/mL (10 ⁹)		
DGCC9209	Control	x	x	236	2.5	236	0.79	0	311
DGCC9209	hemin	Sigma	0,01	176	3.2	228	0.74	-52	424
DGCC9209	Spirulina	SEAH lot 2	1	212	1.6	236	0.67	-24	235
			2	184	2.4	228	0.77	-44	308
			5	176	3.1	228	0.96	-52	325

Table 15

Strain	Sample name	Supplier	Concentration (g/l)	Aerobic conditions		Anaerobic conditions		Delta Ta aerobic -Ta anaerobic (min)	Ratio CFU aerobic / CFU anaerobic (%)
				Ta (min)	CFU/mL (10 ⁹)	Ta (min)	CFU/mL (10 ⁹)		
DGCC4634	Control	x	x	244	0.84	224	0.69	20	123
DGCC4634	hemin	Sigma	0,01	256	0.86	252	0.67	4	129
DGCC4634	Spirulina	SEAH lot 2	1	232	1.0	240	0.74	-8	140
			5	228	1.2	240	0.91	-12	135

Table 16

CLAIMS

1. A method of producing a harvested lactic acid bacteria composition, comprising the steps of

5 (i) admixing a culture medium and a lactic acid bacteria, and an algae or a fraction thereof;

(ii) culturing said lactic acid bacteria admixture under aerobic growth conditions to produce a LAB composition with increased lactic acid bacteria viable active biomass;

wherein said increased viable active biomass of the LAB composition is defined as at least one of the following:

10 a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

15 b) the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof;

(iii) harvesting the LAB from the LAB composition of step ii) by separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed
20 and/or by separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed, to produce a harvested LAB composition.

2. Use of an algae or a fraction thereof, in the manufacture of a lactic acid bacteria composition for increasing viable active biomass of lactic acid bacteria, cultured under
25 aerobic growth conditions, wherein increased viable active biomass is defined as at least one of the following:

a) the lag phase of growth on a fermentable substrate (Ta value) of the lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta

value of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

b) wherein the content of viable cells, expressed in colony forming units (CFU), of the lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a lactic acid bacteria culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof.

3. The method according to claim 1 or the use according to claim 2 wherein in particular said algae or a fraction thereof is a prokaryotic algae, such as a cyanobacteria, or an eukaryotic algae selected from the group consisting of a red algae and a green algae, or any fraction thereof, and more particularly wherein said algae or a fraction thereof is selected from the group consisting of spirulina, chlorella and a fraction thereof.

4. The method or use according to any preceding claim, wherein the Ta value of a lactic acid bacteria composition obtained after culture in the presence of said algae or a fraction thereof under aerobic conditions, is at least 12, at least 15, at least 20, at least 30 or at least 40 minutes less than the Ta value of a lactic acid bacteria culture medium obtained after culture in the absence of said algae or a fraction thereof.

5. The method or use according to any preceding claim, wherein the content of viable cells of a lactic acid bacteria composition obtained after culture in the presence of said algae or a fraction thereof under aerobic conditions, is at least 130, at least 140, at least 150 or at least 200 % the content of viable cells of a lactic acid bacteria culture medium obtained after culture in the absence of said algae or a fraction thereof.

6. The method or use according to any preceding claim, wherein further the optical density (OD) gain of the lactic acid bacteria composition is of at least 120, at least 130, at least 140, at least 150, at least 200 or at least 300 % the OD gain of a lactic acid bacteria culture medium or a same sized sample thereof obtained after culture in the absence of said algae or a fraction thereof.

7. A method according to any one of claims 1 to 6, wherein said harvested LAB composition, when at least 80% of the final culture medium is removed, has an increased lactic acid bacteria viable active biomass,

said increased viable active biomass of the harvested LAB composition is defined as at least one of the following:

5 a) the lag phase of growth on a fermentable substrate (Ta value), of the harvested lactic acid bacteria composition, or a sample thereof, is at least 10 minutes less than the Ta value of a harvested LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof; and/or

10 b) the content of viable cells, expressed in colony forming units (CFU), of the harvested lactic acid bacteria composition, or a sample thereof, is at least 120% the content of viable cells of a harvested LAB culture medium, or a same sized sample thereof, obtained after culture in the absence of said algae or a fraction thereof

15 8. The method according to claim 7 wherein in particular said algae or a fraction thereof is a prokaryotic algae, such as a cyanobacteria, or an eukaryotic algae selected from the group consisting of a red algae and a green algae, or any fraction thereof, and more particularly wherein said algae or a fraction thereof is selected from the group consisting of spirulina, chlorella and a fraction thereof.

20 9. The method according to any one of claims 7 to 8, wherein the Ta value of a harvested lactic acid bacteria composition obtained after culture in the presence of said algae or a fraction thereof under aerobic conditions, is at least 12, at least 15, at least 20, at least 30 or at least 40 minutes less than the Ta value of a harvested lactic acid bacteria culture medium obtained after culture in the absence of said algae or a fraction thereof.

25 10. The method according to any one of claims 7 to 9, wherein the content of viable cells of a harvested lactic acid bacteria composition obtained after culture in the presence of said algae or a fraction thereof under aerobic conditions, is at least 130, at least 140, at least 150 or at least 200 % the content of viable cells of a harvested lactic acid bacteria culture medium obtained after culture in the absence of said algae or a fraction thereof.

30

11. The method or use of any preceding claim wherein:

(i) the Ta value is determined by Assay I as presented herein; and/or

(ii) the CFU is determined by Assay II as presented herein; and/or

(iii) the OD gain is determined by Assay III as presented herein.

5 12. The method or use according to any preceding claim wherein said algae or a fraction thereof is used or is present or is added into said culture medium, at a concentration of at least 0.1 g/L, at least 0.5 g/L, in particular at least 1g/L of culture medium.

10 13 The method or use according to any preceding claim wherein said lactic acid bacteria composition consists of a strain from species *Lactococcus* or a strain from species *Leuconostoc*, or comprises at least a strain from species *Lactococcus* or at least a strain from species *Leuconostoc*.

14. The method or use according to any preceding claim wherein, in calculating the Ta value, the lag phase of growth is carried out on a fermentable substrate which is milk.

15 15. A use according to any of claims 2 to 6 and 7 to 14 wherein lactic acid bacteria composition or lactic acid bacteria is harvested by separating the LAB from the final culture medium, such as at least 80% of the final culture medium is removed and/or by separating the LAB from a portion of the algae or a fraction thereof such that at least 5% of the algae or a fraction thereof initially admixed in step (i) is removed, to produce a harvested LAB composition.

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16. A harvested lactic acid bacteria composition obtained by the method or use of any one of claims 1-15.

25 17. An intermediate starter culture obtained from a harvested lactic acid bacteria composition according to claim 16, after washing, and/or freezing and/or drying and/or formulating said harvested LAB composition of the invention.

18. A harvested lactic acid bacteria composition or an intermediate starter culture according to claim 16 or 17, which is liquid, solid, frozen, dried, freeze-dried, in the form of pellets or frozen pellets, or a powder or dried powder.

19. A frozen intermediate starter culture according to claim 18, which comprises between 0.1% and 20% of remaining algae or a fraction thereof, calculated as weight by weight of the frozen intermediate starter culture.

20. A dried intermediate starter culture obtained by drying the frozen intermediate starter culture according to claim 19.

21. A starter culture comprising at least one intermediate starter culture according to any one of claims 17 to 20, and at least one other intermediate starter culture.

22. Use of a harvested lactic acid bacteria composition, intermediate starter culture or starter culture according to any of claims 16 or 21, for increasing the acidity of a fermentable substrate and/or for producing a fermented product.

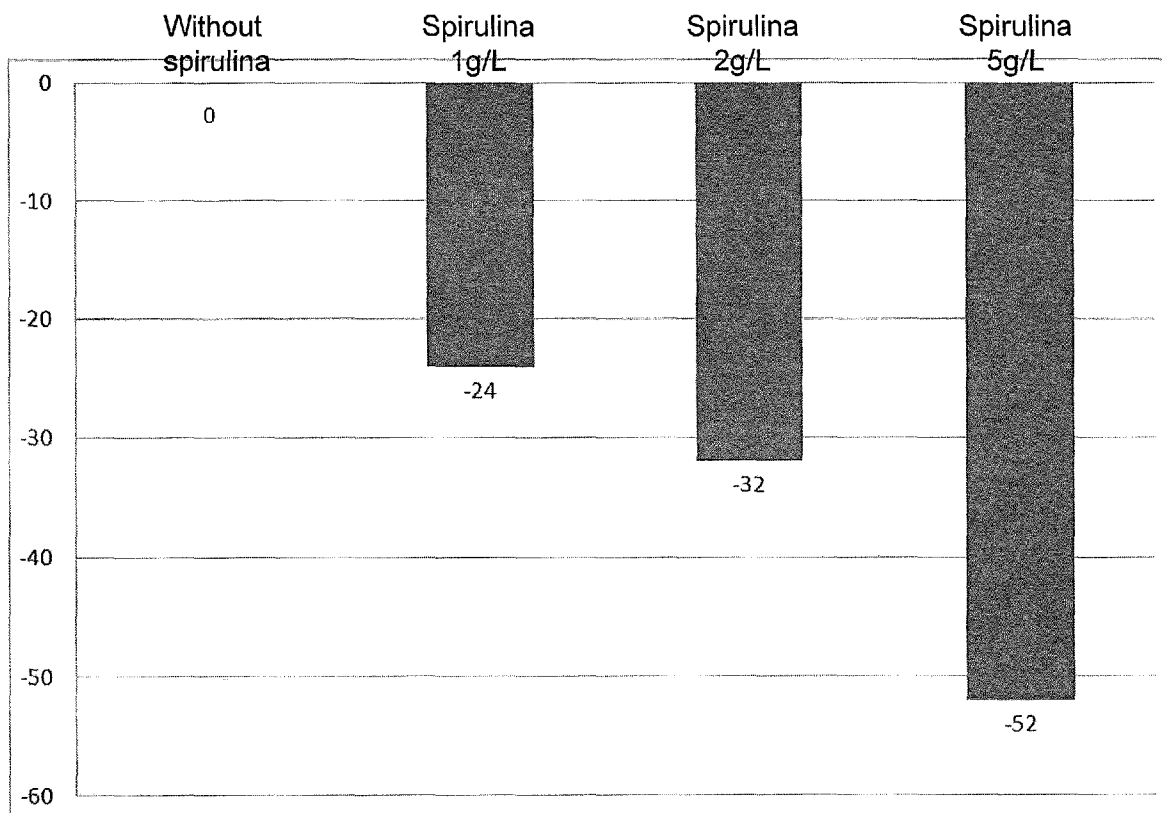


Fig. 1

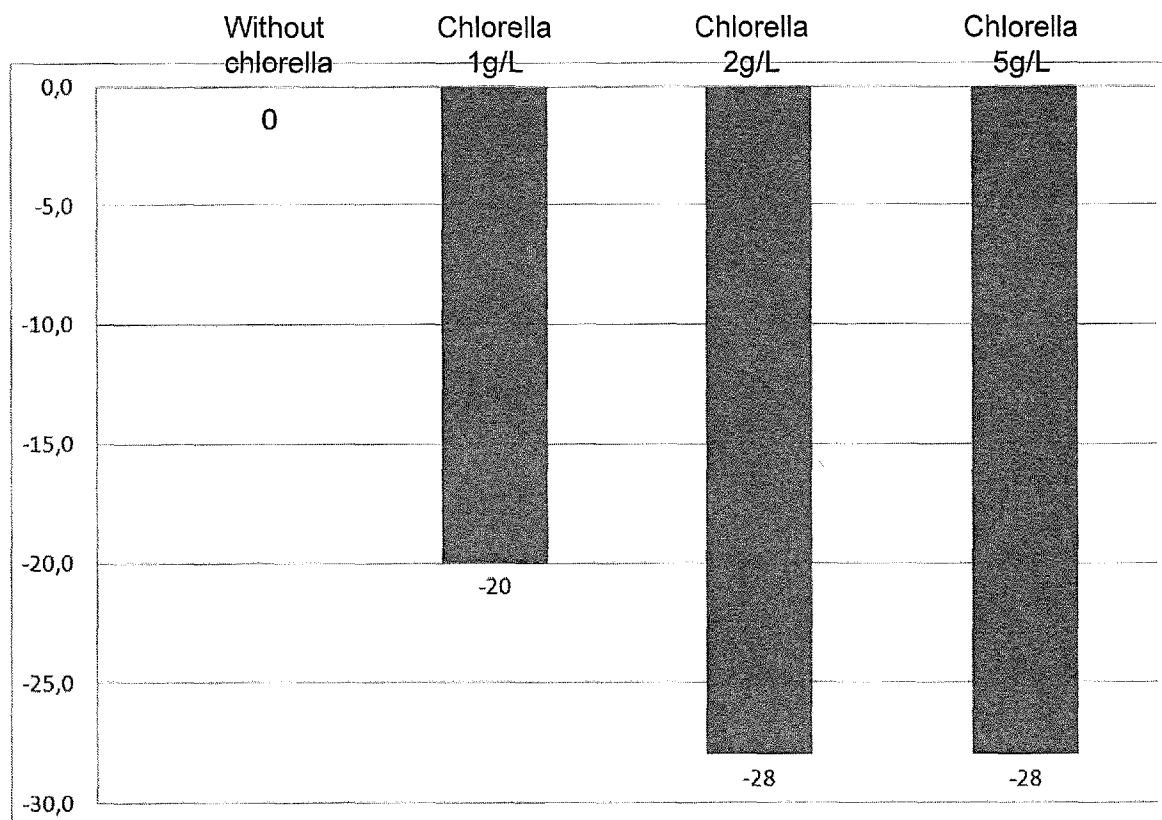
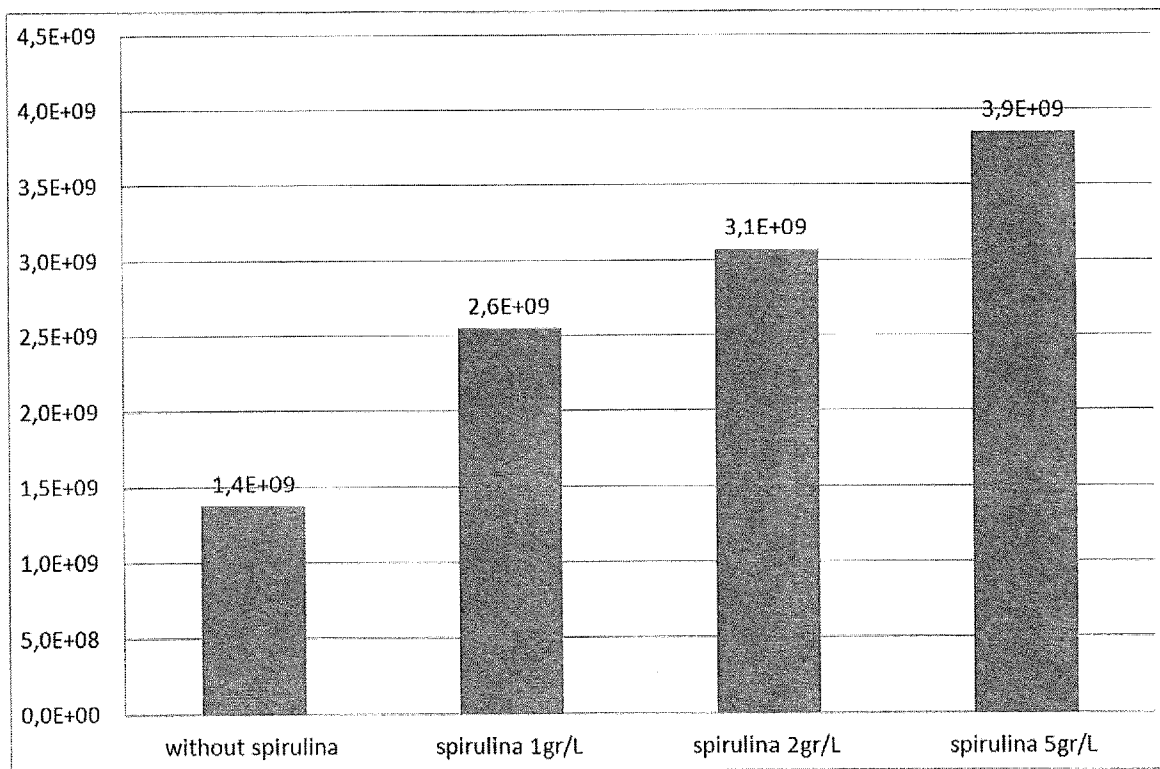
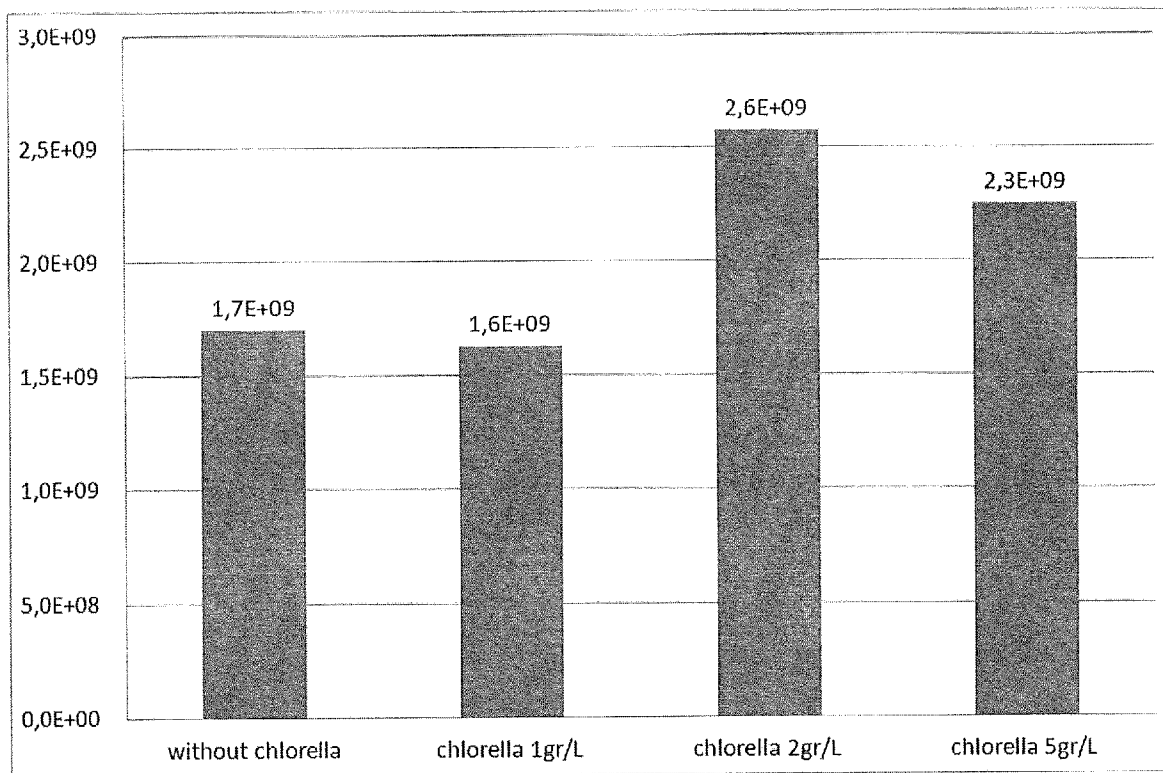
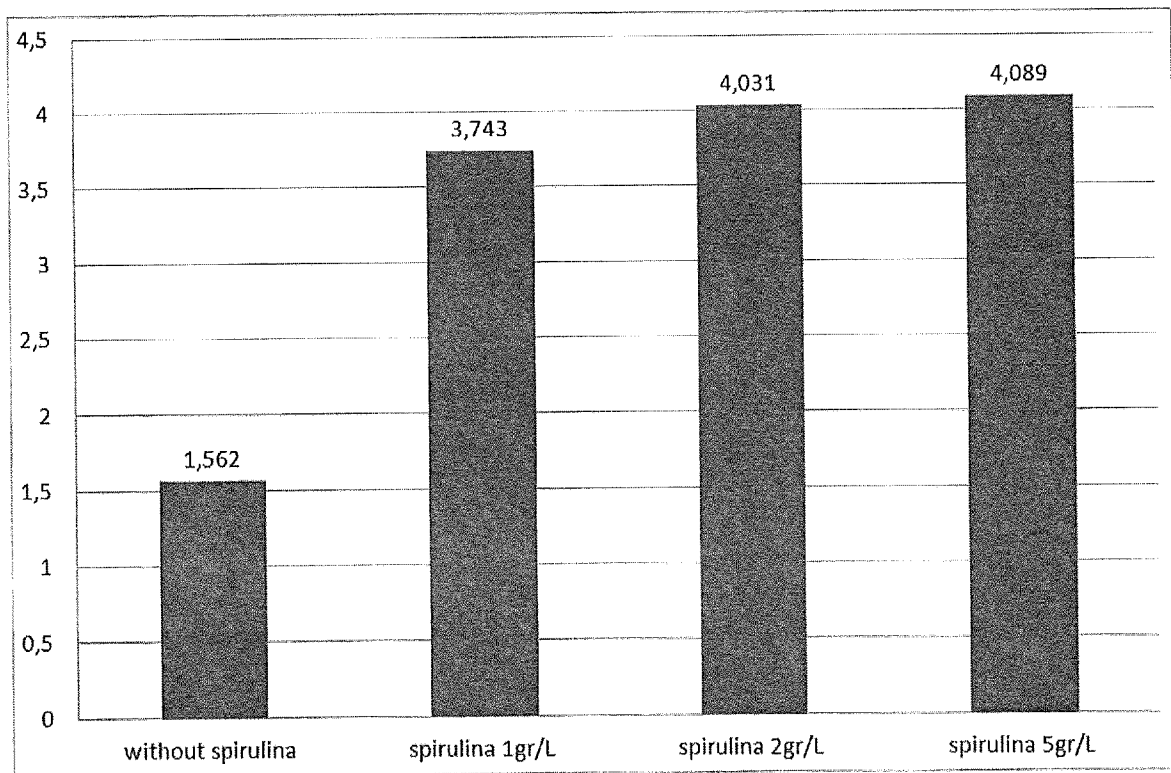
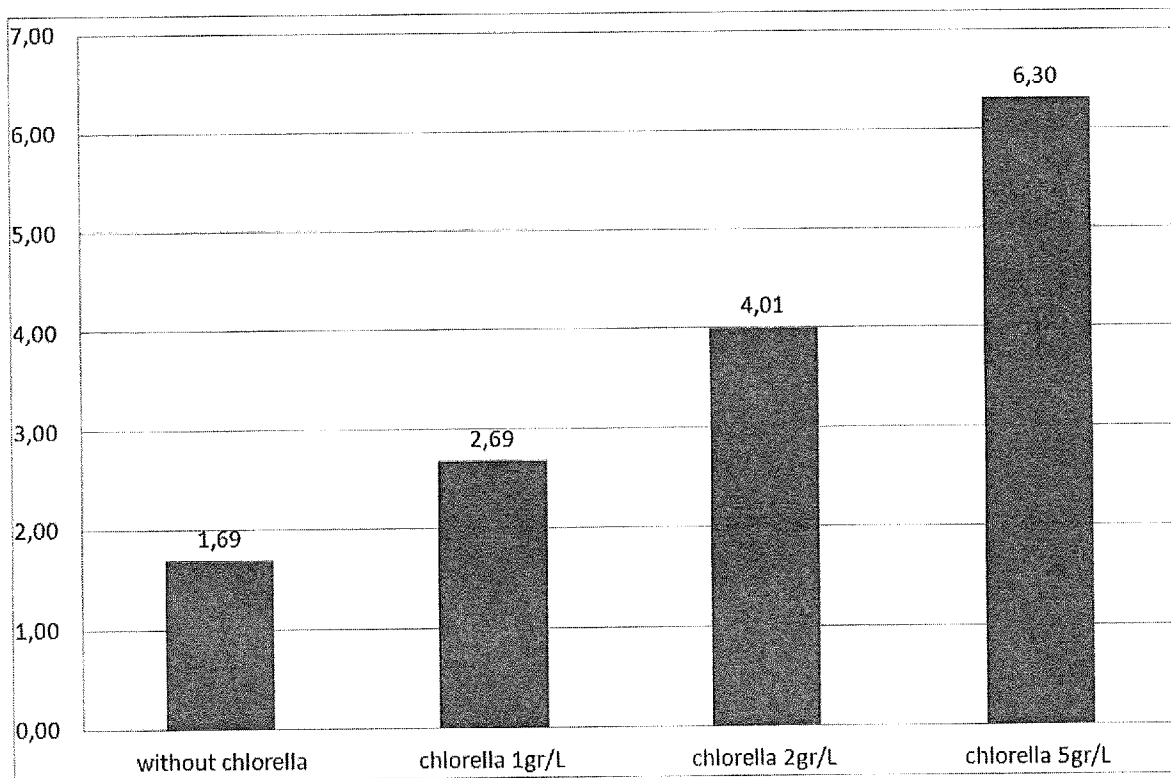


Fig. 2

**Fig. 3****Fig. 4**

**Fig. 5****Fig. 6**

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/073505

A. CLASSIFICATION OF SUBJECT MATTER INV. A23C9/123 C12R1/46 C12N1/04 C12N1/20 C12N1/38 C12P1/00 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 C12N C12P		
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, BIOSIS, FSTA, COMPENDEX		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/210545 A1 (SAKAKIBARA MASAKI [JP] ET AL) 21 September 2006 (2006-09-21) the whole document	1-22
X	----- DOLA BHOWMIK ET AL: "Probiotic Efficiency of Spirulina platensis - Stimulating Growth of Lactic Acid Bacteria", AMERICAN-EURASIAN J. AGRIC. & ENVIRON. SCI., vol. 6, no. 5, 1 January 2009 (2009-01-01), pages 546-549, XP055165380, ISSN: 1818-6769	2-6,11,12,14
Y	the whole document ----- -/-	1-10,15-22
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
4 February 2015		11/02/2015
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 Florey, Marianne

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/073505

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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Y	the whole document	1-10,15-22
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Y	----- J PARADA: "Lactic acid bacteria growth promoters from <i>Spirulina platensis</i> ", INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, vol. 45, no. 3, 22 December 1998 (1998-12-22), pages 225-228, XP055165377, ISSN: 0168-1605, DOI: 10.1016/S0168-1605(98)00151-2 the whole document	1-22
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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	N Tharmaraj ET AL: "Selective Enumeration of Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus, and Propionibacteria", Journal of Dairy Science, 1 July 2003 (2003-07-01), pages 2288-2296, XP055134253, United States DOI: 10.3168/jds.S0022-0302(03)73821-1 Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/pubmed/12906045 the whole document	1-22
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A	ASMAHAN AZHARI ALI: "Beneficial Role of Lactic Acid Bacteria in Food Preservation and Human Health: A Review", RESEARCH JOURNAL OF MICROBIOLOGY, vol. 5, no. 12, 1 January 2010 (2010-01-01), pages 1213-1221, XP55166867, ISSN: 1816-4935 the whole document	1-22

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International application No

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