(12) PATENT (11) Application No. AU 199911547 B2
(19) AUSTRALIAN PATENT OFFICE (10) Patent No. 743435

(54) Title
Novel lactic acid bacteria species

(51)[7] International Patent Classification(s)
C12N 001/00

(21) Application No: **199911547** (22) Application Date: **1998.10.09**

(87) WIPO No: **WO99/20739**

(30) Priority Data

(31) Number	(32) Date	(33) Country
97203245	**1997.10.17**	**EP**

(43) Publication Date : **1999.05.10**
(43) Publication Journal Date : **1999.07.08**
(44) Accepted Journal Date : **2002.01.24**

(71) Applicant(s)
Societe Des Produits Nestle S.A.

(72) Inventor(s)
Walter Gaier; David Pridmore; Francesca Stingele; Jean-Richard Neeser; Patrice Desachy; Bruno Pot

(74) Agent/Attorney
BALDWIN SHELSTON WATERS,Level 21,60 Margaret Street,SYDNEY NSW 2000

(56) Related Art
WO 87/06267
WILLERS JMN ET AL, ANTONIE VAN LEEUWENKOEK, 1973, 39:609-617

OPI DATE 10/05/99 APPLN. ID 11547/99
AOJP DATE 08/07/99 PCT NUMBER PCT/EP98/06636

AU9911547

D... ...TS (PCT)

(51) Classification internationale des brevets 6 : C12N 1/00 | A2

(11) Numéro de publication internationale: **WO 99/20739**

(43) Date de publication internationale: 29 avril 1999 (29.04.99)

(21) Numéro de la demande internationale: PCT/EP98/06636

(22) Date de dépôt international: 9 octobre 1998 (09.10.98)

(30) Données relatives à la priorité:
97203245.2 17 octobre 1997 (17.10.97) EP

(71) Déposant *(pour tous les Etats désignés sauf US)*: SOCIETE DES PRODUITS NESTLE S.A. [CH/CH]; Case postale 353, CH-1800 Vevey (CH).

(72) Inventeurs; et
(75) Inventeurs/Déposants *(US seulement)*: GAIER, Walter [DE/FR]; Le Gascoin, Fourneville, F-14600 Honfleur (FR). PRIDMORE, David [GB/CH]; 126, route du Jorat, Vers-chez-les-Blancs, CH-1000 Lausanne 26 (CH). STINGELE, Francesca [DE/CH]; 10, route du Signal, CH-1018 Lausanne (CH). NEESER, Jean-Richard [CH/CH]; 6, sentier de Courtaraye, CH-1073 Savigny (CH). DESACHY, Patrice [CH/CH]; 2C, chemin du Fau-Blanc, CH-1009 Pully (CH). POT, Bruno [BE/BE]; Ter Beke 23, B-8200 Sint-Michiels Brugge (BE).

(74) Mandataire: VUILLE, Roman, L.; 55, avenue Nestlé, CH-1800 Vevey (CH).

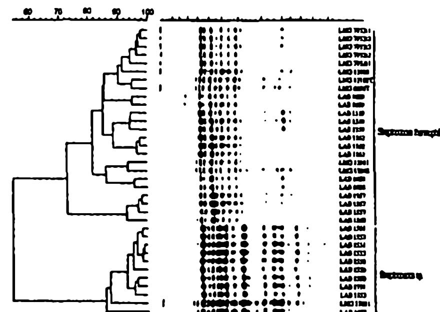
(81) Etats désignés: AU, BG, BR, CA, CN, CZ, HU, ID, IL, JP, KR, MX, NO, NZ, PL, SG, TR, US, brevet ARIPO (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), brevet eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Publiée
Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.

(54) Title: NOVEL LACTIC ACID BACTERIA SPECIES

(54) Titre: NOUVELLE ESPECE DE BACTERIE LACTIQUE

(57) Abstract



The invention concerns a lactic acid bacteria strain (1), whereof the RNA ribosome 16S is characteristic of the genus *Streptococcus* (2), whose profile in crude proteins, obtained after migration of crude proteins on SDS-PAGE gel electrophoresis, is characteristic of that of the CNCM I-1926 lactic acid bacteria strain, but distinct from those of species recognised as belonging to the genus *Streptococcus*, namely *S. acidominimus, S.agalactiae, S. alactolyticus, S. anginosus, S. bovis, S. canis, S. caprinus, S. constellatus, S. cricetus, S. cristatus, S. difficile, S. downei, S. dysgalactiae ssp. dysgalactiae, S. dysgalactiae ssp. equisimilis, S. equi, S. equi ssp. equi, S. equi ssp. zooepidemicus, S. equinus, S.ferus, S. gallolyticus, S. gordonii, S. hyointestinalis, S. hyovaginalis, S. iniae, S. intermedius, S. intestinalis, S. macacae, S. mitis, S. mutans, S. oralis, S. parasanguinis, S. parauberis, S. phocae, S. pleomorphus, S. pneumoniae, S. porcinus, S. pyogenes, S. ratti, S. salivarius, S. sanguinis, S. shiloi, S. sobrinus, S. suis, S. thermophilus, S. thoraltensis, S. uberis, S. vestibularis, S. viridans*. The invention also concerns the use of said lactic acid bacteria strain, or a polysaccharide secreted by said strain, for preparing a food composition, in particular a lactic milk or fresh cheese. The invention further concerns a food or pharmaceutical composition comprising a lactic acid bacteria or a polysaccharide constituted by a chain of glucose, galactose and N-acetylglucosamine in a ratio of 3:2:1 respectively.

Novel Lactic Acid Bacteria Species

The present invention relates to a new species of lactic acid bacterium belonging to the genus
5 *Streptococcus*.

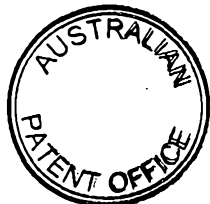
State of the art

The identification of lactic acid bacteria is
10 essential in the dairy industry, and consists in differentiating, between several species, distinctive characteristics of a morphological, physiological and/or genetic nature.

The distinctive physiological characters for a
15 given species of lactic acid bacterium may be obtained by means of various tests including, for example, the analysis of the capacity to ferment various sugars and the standard analysis of the migration profile of total proteins on an SDS-PAGE type electrophoresis gel (Pot
20 *et al.*, Taxonomy of lactic acid bacteria, *in* Bacteriocins of lactic acid bacteria, Microbiology, Genetics and Applications, L. De Vuyst, and E.J. Vandamme ed., Blackie Academic & Professional, London, 1994).

25 The migration profile of the total proteins of a given species, obtained on an SDS-PAGE electrophoresis gel, when it is compared with the aid of a densitometer with other profiles obtained from other species, makes it possible to determine the
30 taxonomic relationships between the species. Numerical analysis of the various profiles, for example with the GelCompar® software makes it possible in particular to establish a degree of correlation between the species which is a function of various parameters, in
35 particular of the algorithms used (GelCompar, version 4.0, Applied Maths, Kortrijk, Belgium; algorithms: "Pearson Product Moment Correlation Coefficient, Unweighted Pair Group Method Using Average Linkage").

To date, comparative analysis of the total
40 protein profile by electrophoresis on an SDS-PAGE gel

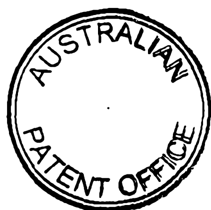


has been thoroughly tested as an effective means for distinguishing between homogeneous and distinct groups of species of lactic acid bacteria (Pot *et al.*, Chemical Methods in Prokaryotic Systematics, Chapter 14, M. Goodfellow, A.G. O'Donnell, Ed., John Wiley & Sons Ltd, 1994).

With this SDS-PAGE method, the preceding experiments have thus shown that when a degree of Pearson correlation of more than 78 (on a scale of 100) is obtained between two strains of lactic acid bacteria, it is possible justifiably to deduce therefrom that they belong to the same species (Kersters *et al.*, Classification and Identification methods for lactic bacteria with emphasis on protein gel electrophoresis, *in* Acid Lactic Bacteria, Actes du Colloque Lactic '91, 33-40, Adria Normandie, France, 1992; Pot *et al.*, The potential role of a culture collection for identification and maintenance of lactic acid bacteria, Chapter 15, pp. 81-87, *in*: The Lactic Acid Bacteria, E.L. Foo, H.G. Griffin, R. Mollby and C.G. Heden, Proceedings of the first lactic computer conference, Horizon Scientific Press, Norfolk).

By way of example, it has recently been possible to divide the group of acidophilic lactic acid bacteria into 6 distinct species by means of this technique (Pot *et al.*, J. General Microb., 139, 513-517, 1993). Likewise, this technique has recently been able to establish, in combination with other techniques, the existence of several new species of *Streptococcus*, such as *Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus hyovaginalis* sp. *nov.* and *Streptococcus thoraltensis* sp. *nov* (Vandamme et *al.*, Int. J. Syst. Bacteriol., 46, 774-781, 1996; Devriese et *al.*, Int. J. Syst. Bacteriol., 1997, In press).

The identification of new species of lactic acid bacteria cannot however be reduced to a purely morphological and/or physiological analysis of the bacteria. Indeed, two species which are very closely



related morphologically and/or physiologically may be distantly related from a genetic point of view. Analysis of the 16S ribosomal RNA of the lactic acid bacteria is thus of vital importance for determining definitively if a lactic acid bacterium belongs to a genus or a species already known.

5 To date, the "Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH" (DSM, Braunschweig, Germany) has officially recorded about 48 different species belonging to the genus *Streptococcus* (see the list below). All these species possess a 16S ribosomal RNA which is typical of the genus *Streptococcus*, and may be divided into distinct and homogeneous groups by means of the SDS-PAGE technique 10 mentioned above.

The present invention relates to the identification, by means of the identification techniques presented above, of a new species of lactic acid bacterium belonging to the genus *Streptococcus*, and to its use in the dairy industry in general.

Any discussion of the prior art throughout the specification should in no way be 15 considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Summary of the Invention

According to a first aspect, the present invention provides strain of lactic acid bacterium,

20 - whose 16S ribosomal RNA is characteristic of the genus *Streptococcus*,

- whose total protein profile, obtained after culture of the bacterium in an MRS medium for 24 h at 28°C, extraction of the total proteins and migration of the proteins on an SDS-PAGE electrophoresis gel, exhibits a degree of Pearson correlation of at least 78 with respect to the profile obtained under identical conditions with the strain of lactic 25 acid bacterium CNCM I-1920, but distinct from those of the recognized species

belonging to the genus *Streptococcus*, namely *S. acidominimus*, *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. bovis*, *S. canis*, *S. caprinus*, *S. constellatus*, *S. cricetus*, *S. cristatus*, *S. difficile*, *S. downei*, *S. dysgalactiae ssp. dysgalactiae*, *S. dysgalactiae ssp. equisimilis*, *S. equi*, *S. equi ssp. equi*, *S. equi ssp. zooepidemicus*, *S. equinus*, *S. ferus*, *S. gallolyticus*, *S. gordonii*, *S. hyointestinalis*, *S. hyovaginalis*, *S. iniae*, *S. intermedius*, *S. intestinalis*, *S. macacae*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. parauberis*, *S. phocae*, *S. pleomorphus*, *S. pneumoniae*, *S. porcinus*, *S. pyogenes*, *S. ratti*, *S. salivarius*, *S. sanguinis*, *S. shiloi*, *S. sobrinus*, *S. suis*, *S. thermophilus*, *S. thoraltensis*, *S. uberis*, *S. vestibularis*, *S. viridans*.

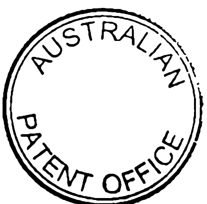
According to a second aspect, the present invention provides use of a strain of lactic acid bacterium according to the first aspect for the preparation of a dietary composition, in particular an acidified milk.

The dietary composition may be a fromage frais, for example.

The invention also relates to the use of a polysaccharide, capable of being secreted by a lactic acid bacterium according to the invention, which consists of a succession of glucose, galactose and N-acetylglucosamine in a respective proportion of 3:2:1, for the preparation of a dietary or pharmaceutical composition.

Accordingly, a third aspect of the present invention provides use of a polysaccharide, capable of being secreted by a strain of lactic acid bacterium according to the first aspect, for the preparation of a dietary composition.

According to a fourth aspect, the present invention provides a dietary or pharmaceutical composition comprising a strain of lactic acid bacterium according to the first aspect.



According to a fifth aspect, the present invention provides a dietary or pharmaceutical composition comprising a polysaccharide, capable of being secreted by the strain CNCM I-1924, consisting exclusively of a succession of glucose, galactose and N-acetylglucosamine in a respective proportion of 3:2:1.

5 According to a sixth aspect, the present invention provides a polysaccharide, capable of being secreted by the strain CNCM I-1924, consisting exclusively of a succession of glucose, galactose and N-acetylglucosamine in a respective proportion of 3:2:1.

Unless the context clearly requires otherwise, throughout the description and the 10 claims, the words ‘comprise’, ‘comprising’, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

Detailed Description of the Invention

The attachment of the new species according to the invention to the genus 15 *Streptococcus* is preferably demonstrated by comparing the nucleotide sequence of the 16S ribosomal RNA of the bacteria according to the invention, or of their genomic DNA which is transcribed into 16S ribosomal RNA, with those of other genera and species of lactic acid bacteria known to date.

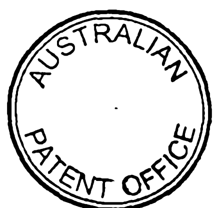
More particularly, it is possible to use the method disclosed in Example 1 below, 20 or alternatively other methods known to a person skilled in the art

(Schleifer et al., System. Appl. Microb., 18, 461-467, 1995; Ludwig et al., System. Appl. Microb., 15, 487-501, 1992), for example. The nucleotide sequence SEQ ID NO: 1 presented in the sequence listing below is
5 characteristic of this new species, and exhibits striking similarities with the 16S ribosomal RNA sequences found in the species of *Streptococcus* recognized to date.

The new species according to the invention,
10 which constitutes a distinct and homogeneous new group, can also be differentiated from the other known species belonging to the genus *Streptococcus* by means of the technique for identification of the total proteins on an SDS-PAGE electrophoresis gel, described above.

15 In particular, this new species may give a total protein profile, obtained after culture of the bacterium in an MRS medium for 24 h at 28°C, extraction of the total proteins and migration of the proteins on an SDS-PAGE electrophoresis gel, which exhibits a
20 degree of Pearson correlation of at least 78 (on a scale of 100) with the profile obtained under identical conditions with the strain of lactic acid bacterium CNCM I-1920, and this with respect to the profiles obtained under identical conditions with a few of the
25 various species of lactic acid bacteria, in particular those indicated below, for example.

More particularly, this technique consists in (1) isolating all the proteins (= total proteins) of a culture of lactic acid bacterium cultured under defined
30 conditions, (2) separating the proteins by electrophoresis on an SDS-PAGE gel, (3) analysing the arrangement of the different protein fractions separated with the aid of a densitometer which measures the intensity and the location of each band, (4) and
35 comparing the protein profile thus obtained with those of several other species of *Streptococcus* which have been obtained, in parallel or beforehand, exactly under the same operating conditions.



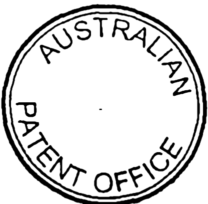
The techniques for preparing a total protein profile as are described above, as well as the numerical analysis of such profiles, are well known to a person skilled in the art. However, the results are only reliable insofar as each stage of the process is sufficiently standardized. Faced with this requirement, standardized procedures are regularly made available to the public by their authors. There may be mentioned in particular that of Pot *et al.* presented during a "workshop" organized by the European Union, at the University of Ghent, in Belgium, on 12 to 16 September 1994 (Fingerprinting techniques for classification and identification of bacteria, SDS-PAGE of whole cell protein).

The software used in the technique for identification on an SDS-PAGE electrophoresis gel is of crucial importance since the degree of correlation between the species depends on the parameters and the algorithms used by this software. Without wishing to go into the theoretical details, quantitative comparison of bands captured by a densitometer and normalized by a computer is preferably made with the Pearson correlation coefficient. The similarity matrix thus obtained may be organized with the aid of the UPGMA (unweighted pair group method using average linkage) algorithm, an algorithm which not only makes it possible to group together the most similar profiles, but also to construct dendogrammes (see K. Kersters, Numerical methods in the classification and identification of bacteria by electrophoresis, *in* Computer-assisted Bacterial Systematics, 337-368, M. Goodfellow, A.G. O'Donnell Ed., John Wiley and Sons Ltd, 1985).

Preferably, the strains of the new species exhibit a total protein profile having a degree of Pearson correlation of at least 85 with respect to one of the strains of bacteria of the new species. For the biotypes mentioned below, this degree of Pearson correlation can even exceed 90, for example.

By means of this technique of identification on an SDS-PAGE electrophoresis gel, the new species belonging to the genus *Streptococcus* according to the invention may be distinguished from all the species of
5 *Streptococcus* recognized to date, namely the species *S. acidominimus, S. agalactiae, S. alactolyticus, S. anginosus, S. bovis, S. canis, S. caprinus, S. constellatus, S. cricetus, S. cristatus, S. difficile, S. downei, S. dysgalactiae ssp. dysgalactiae, S.*
10 *dysgalactiae ssp. equisimilis, S. equi, S. equi ssp. equi, S. equi ssp. zooepidemicus, S. equinus, S. ferus, S. gallolyticus, S. gordonii, S. hyointestinalis, S. hyovaginalis, S.iniae, S. intermedius, S. intestinalis, S. macacae, S. mitis, S. mutans, S. oralis, S.*
15 *parasanguinis, S. parauberis, S. phocae, S. pleomorphus, S. pneumoniae, S. porcinus, S. pyogenes, S. ratti, S. salivarius, S. sanguinis, S. shiloi, S. sobrinus, S. suis, S. thermophilus, S. thoraltensis, S. uberis, S. vestibularis, S. viridans.*

20 The new species according to the invention can also be distinguished by this technique from the lactic acid bacteria which had been previously classified in error in the genus *Streptococcus* such as *S. adjacens* (new classification = *Abiotrophia adiacens*), *S.*
25 *casseliflavus (=Enterococcus casseliflavus), S. cecorum (=Enterococcus cecorum), S. cremoris (=Lactococcus lactis subsp. cremoris), S. defectivus (=Abiotrophia defectiva), S. faecalis (=Enterococcus faecalis), S. faecium (=Enterococcus faecium), S. gallinarum*
30 *(=Enterococcus gallinarum), S. garvieae (=Lactococcus garvieae), S. hansenii (=Ruminococcus hansenii), S. lactis (=Lactococcus lactis subsp. lactis), S. lactis cremoris (=Lactococcus lactis subsp. cremoris), S. lactis diacetilactis (=Lactococcus lactis subsp.*
35 *lactis), S. morbillorum (=Gemella morbillorum), S. parvulus (=Atopobium parvulum), S. plantarum (=Lactococcus plantarum), S. raffinolactis (=Lactococcus raffinolactis)* and *S. saccharolyticus (=Enterococcus saccharolyticus).*



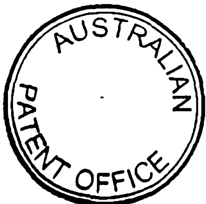
The lactic acid bacteria according to the invention have a characteristic morphology of the *Lactococcus lactis*, for example; that is to say that they have the shape of cocci assembled into chains.

5 The sugars which can be fermented by the new species are generally at least one of the following, namely, D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-(D)-glucosamine, salicin, cellobiose, maltose, lactose, sucrose and raffinose.

10 Among all the strains of the new species which have been isolated in dairies in Switzerland, 7 were deposited under the treaty of Budapest, by way of example, in the Collection Nationale de Culture de Microorganismes (CNCM), 25 rue du docteur Roux, 75724 15 Paris, on 14 October 1997, where they were attributed the deposit numbers CNCM I-1920, I-1921, I-1922, I-1923, I-1924, I-1925 and I-1926.

The strains of the new species can be used to prepare a dietary or pharmaceutical product, in 20 particular in the form of a fresh, concentrated or dried culture, for example.

Milk-based products are obviously preferred within the framework of the invention. Milk is however understood to mean, on the one hand, a milk of animal 25 origin, such as cow's, goat's, sheep's, buffaloe's, zebra's, horse's, ass's or camel's milk, and the like. This milk may be a milk in the native state, a reconstituted milk, a skimmed milk or a milk supplemented with compounds necessary for the growth of 30 the bacteria or for the subsequent processing of fermented milk, such as fat, proteins of a yeast extract, peptone and/or a surfactant, for example. The term milk also applies to what is commonly called a vegetable milk, that is to say an extract of plant 35 materials which have been treated or otherwise, such as leguminous plants (soya bean, chick pea, lentil and the like) or oilseeds (colza, soya bean, sesame, cotton and the like), which extract contains proteins in solution or in colloidal suspension, which are coagulable by

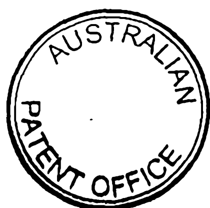


chemical action, by acid fermentation and/or by heat. Finally, the word milk also denotes mixtures of animal milks and of vegetable milks.

Pharmaceutical products may be all sorts of products intended to be administered orally, or even topically, which comprise an acceptable pharmaceutical carrier to which, or onto which, a culture of the new species is added in fresh, concentrated or dried form, for example. These pharmaceutical products may be provided in the form of an ingestible suspension, a gel, a diffuser, a capsule, a hard gelatin capsule, a syrup, or in any other galenic form known to persons skilled in the art.

Moreover, some strains of the new species according to the invention, representing a new biotype of this species, may also have the remarkable property of being both mesophilic and thermophilic (mesophilic/thermophilic biotype). The strains belonging to this biotype indeed have a growth optimum from about 28°C up to about 45°C. This property can be easily observed (1) by preparing several cultures of a mesophilic/thermophilic biotype, in parallel, at temperatures ranging from 20 to 50°C, (2) by measuring the absorbance values for the media after 16 h of culture, for example, and (3) by grouping the results in the form of a graph representing the absorbance as a function of the temperature (graditherm). Figure 1 is particularly representative of the graphs which can be obtained with this type of mesophilic/thermophilic biotype according to the invention. As a guide, among the strains of the new species having this particular biotype, the strains CNCM I-1920, I-1921 and I-1922 are particularly representative, for example.

The use of a mesophilic/thermophilic biotype in the dairy industry is of great importance. Indeed, this species may be used for the preparation of mesophilic or thermophilic starters. It is thus possible to industrially produce acidified milks at 45°C in order to obtain a "yoghurt" type product. It is also possible



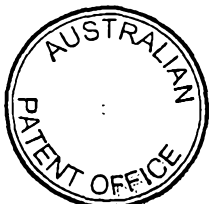
to industrially produce fromage frais by fermenting a milk in the presence of rennet at 28°C, and separating therefrom the curd thus formed by centrifugation or ultrafiltration. The problems of clogging of the machines linked to the use of thermophilic ferments are thus eliminated (these problems are disclosed in patent application EP No. 96203683.6).

Moreover, other strains of the new species according to the invention, representing another new biotype of this species, may exhibit the remarkable property of conferring viscosity to the fermentation medium (texturing biotype). The viscous character of a milk fermented by a texturing biotype according to the invention may be observed and determined as described below.

1. By observing the structure of a milk acidified by a texturing biotype in comparison with that of milk acidified by non-texturing cultures. The non-viscous milk adheres to the walls of a glass cup, whereas the viscous milk is self-coherent.

2. Another test may be carried out using a pipette. The pipette is immersed in the acidified milk which is sucked up in a quantity of about 2 ml, and then the pipette is withdrawn from the milk. The viscous milk forms a rope between the pipette and the liquid surface, whereas the non-viscous milk does not give rise to this phenomenon. When the liquid is released from the pipette, the non-viscous milk forms distinct droplets just like water, whereas the viscous milk forms droplets ending with long ropes which go up to the tip of the pipette.

3. When a test tube filled up to roughly a third of a rotary shaker, the non-viscous milk climbs



up the inner surface of the wall, whereas the rise of the viscous milk is practically zero.

The viscous character of this particular biotype may also be determined with the aid of a rheological parameter measuring the viscosity. A few commercial apparatus are capable of determining this parameter, such as the rheometer Bohlin VOR (Bohlin GmbH, Germany). In accordance with the manufacturer's instructions, the sample is placed between a plate and a truncated cone of the same diameter (30 mm, angle of 5.4°, gap of 0.1 mm), then the sample is subjected to a continuous rotating shear rate gradient which forces it to flow. The sample, by resisting the strain, develops a tangential force called shear stress. This stress, which is proportional to the flow resistance, is measured by means of a torsion bar. The viscosity of the sample is then determined, for a given shear rate, by the ratio between the shear stress (Pa) and the shear rate (s^{-1}) (see also "Le Technoscope de Biofutur", May 97).

The tests of rheological measurement of the texturing character of this biotype have led to the following definition. A lactic acid bacterium belonging to the texturing biotype according to the invention is a bacterium which, when it ferments a semi-skimmed milk at 38°C up to a pH of 5.2, gives to the medium a viscosity which is greater than 100 mPa.s at a shear rate of the order of 293 s^{-1}, for example. As a guide, the strains CNCM I-1922, I-1923, I-1924, I-1925 and I-1926 are particularly representative of this texturing biotype for example.

This texturing biotype is also of great importance in the dairy industry because its capacity to give viscosity to a dairy product is exceptionally high when it is compared with those of other species of texturing lactic acid bacteria, in particular with the strains *Lactobacillus helveticus* CNCM I-1449, *Streptococcus thermophilus* CNCM I-1351, *Streptococcus*

thermophilus CNCM I-1879, *Streptococcus thermophilus* CNCM I-1590, *Lactobacillus bulgaricus* CNCM I-800 and *Leuconostoc mesenteroides ssp. cremoris* CNCM I-1692, which are mentioned respectively in patent applications
5 EP699689, EP638642, EP97111379.0, EP750043, EP367918 and EP97201628.1.

It is also possible to note that the production of a viscosity may also take place, for some strains, in a very broad temperature range which extends from
10 the mesophilic temperatures (25-30°C) to the thermophilic temperatures (40-45°C). This characteristic feature represents an obvious technological advantage.

However, some strains belonging to this new
15 texturing biotype produce, moreover, an exopolysaccharide (EPS) of high molecular weight whose sugar composition is similar to that found in the oligosaccharides in human breast milk. The EPS in fact consists of a succession of glucose, galactose and N-
20 acetylglucosamine in a proportion of 3:2:1 respectively (A. Kobata, *in* the Glycoconjugates, Vol. 1, "Milk glycoproteins and oligosaccharides", p. 423-440, Ed. I. Horowitz and W. Pigman, Ac. Press, N.Y., 1977). As a guide, the strains CNCM I-1923, I-1924, I-1925 and I-
25 1926 produce this polysaccharide.

This exopolysaccharide, in native or hydrolysed form, could thus advantageously satisfy a balanced infant diet.

For that, it is possible to prepare a diet for
30 children and/or breast-feeding infants comprising a milk which has been acidifed with at least one strain of lactic acid bacterium producing an EPS consisting of a succession of glucose, galactose and N-acetylglucosamine in a proportion of 3:2:1,
35 respectively, in particular with the strains CNCM I-1924, I-1925 or I-1926, for example.

It is also possible to isolate this EPS beforehand from a culture medium of this biotype, and

to use it, in native or hydrolysed form, as ingredient in an infant diet, for example.

The isolation of the EPS generally consists in removing the proteins and the bacteria from the culture medium and in isolating a purified fraction of the EPS. It is also possible to carry out the extraction of the proteins and of the bacteria by precipitation with an alcohol or trichloroacetic acid followed by centrifugation, while the EPS can be purified by precipitation in a solvent such as acetone followed by centrifugation, for example. If necessary, the EPS may also be purified by means of a gel-filtration or affinity chromatography, for example.

In the context of the present invention, the isolation of an EPS also covers all the methods of production of an EPS by fermentation followed by concentration of the culture medium by drying or ultrafiltration, for example. The concentration may be performed by any method known to a person skilled in the art, and in particular by freeze-drying or spray-drying in a stream of hot air, for example. To this effect, the methods described in US 3,985,901, EP 298605 and EP 63438 are incorporated by reference into the description of the present invention.

Insofar as the maternal oligosaccharides are small in size, it may be advantageous to carry out beforehand a partial hydrolysis of the EPS according to the invention. Preferably, the hydrolysis conditions are chosen so as to obtain oligosaccharides having 3 to 10 units of sugar, that is to say therefore oligosaccharides having a molecular weight of the order of 600 to 2000 Dalton, for example.

More particularly, it is possible to hydrolyse the EPS according to the invention in a 0.5 N trifluoroacetic acid (TFA) solution for 30-90 min at 100°C, and then to evaporate the TFA and to recover the oligosaccharides.

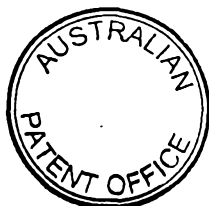
A preferred infant product comprises hydrolysed protein material of whey from which allergens, chosen

from a group of allergens consisting of α-lactalbumin, β-lactoglobulin, serum albumin and the immunoglobulins, have not been removed and in which the hydrolysed protein material, including the hydrolysed allergens, exist in the form of hydrolysis residues having a molecular weight not greater than about 10,000 Dalton, such that the hydrolysed material is substantially free of allergenic proteins and of allergens of protein origin (= hypoallergenic product in accordance with European Directive 96/4/EC; Fritsche *et al.*, Int. Arch. Aller and Appl. Imm., 93, 289-293, 1990).

It is possible to mix the EPS according to the invention, in native or partially hydrolysed form, with this hydrolysed protein material of whey, and to then incorporate this mixture, in dried form or otherwise, into numerous food preparations for dietetic use, in particular into foods for breast-feeding infants and into foods which can be easily absorbed, intended primarily for people suffering from allergies, for example.

The present invention is described in greater detail by the examples presented below, which are preceded by a brief description of the figures. It goes without saying however, that these examples are given by way of illustration of the subject of the invention and do not constitute in any manner a limitation thereto. The percentages are given by weight unless otherwise stated.

- Figure 1 represents a photograph of migration profiles of the total proteins of several strains of the new species, on an SDS-PAGE electrophoresis gel, in comparison with those obtained with *Streptococcus thermophilus* strains. The degree of filiation of the strains is indicated with the aid of the Pearson correlation scale and by means of a tree opposite the protein profiles (the degrees of Pearson correlation of 55 to 100 are represented).



- Figure 2 represents the graditherm for the strain CNCM I-1920.

Example 1 Identification of a new species of *Streptococcus*

5

Several strains of lactic acid bacteria isolated from various dairies in Switzerland were the subject of a genetic and physiological identification as follows. The methods used as well as the results obtained, which are represented below, show that these strains are part of a new *Streptococcus* group which is sufficiently distinct and homogeneous for it to be designated as grouping together a new species of lactic acid bacterium. By way of example, some strains belonging to this new species were deposited under the treaty of Budapest in the Collection Nationale de Culture de Microorganismes (CNCM), 25 rue du docteur Roux, 75724 Paris, on 14 October 1997, where they received the identification Nos. CNCM I-1920, I-1921, I-1922, I-1923, I-1924, I-1925 and I-1926.

10

15

20

1. Morphology of the strains isolated: A morphology characteristic of the *Lactococcus lactis*, that is to say a shape of cocci assembled into chains, is observed under a microscope.

25

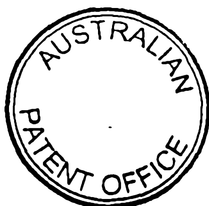
2. Sugar fermentation profile of the strains isolated: The sugars which can be fermented by the strains isolated are generally D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-(D)-glucosamine, salicin, cellobiose, maltose, lactose, sucrose and raffinose. This fermentation profile is similar to that obtained with the species *Lactococcus lactis*.

30

35

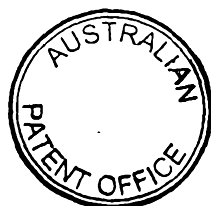
3. 16S ribosomal RNA of the strains isolated

The strains isolated are cultured in 40 ml of HJL medium at 37°C for 24 h, the bacteria are harvested by centrifugation, each bacterial pellet is resuspended in 2.5 ml of TE buffer (10 mM Tris PH 8, 0.1 mM EDTA)



containing 10 mg/ml of lysozyme, and the whole is incubated at 37°C for 1 h. 100 µl of a solution containing 10 mg/ml of proteinase K, 250 µl of a solution containing 500 mM EDTA pH 8.0, and 500 µl of a solution containing 10% SDS are then added. The whole is incubated at 60°C for 1 h so as to ensure complete lysis of the bacteria. After having cooled the mixtures, 2.5 ml of phenol/chloroform are added to them, they are centrifuged for 10 min in a Heraeus centrifuge so as to separate 2 phases, and the top phase is removed. The chromosomal DNA present in the bottom phase is precipitated by addition of 2.5 ml of a solution containing 96% ethanol, and the mixture is gently stirred until a precipitate is formed. The precipitated DNA is removed with the aid of a wooden toothpick, deposited in a 2 ml Eppendorf tube containing 1 ml of a Tris buffer (10 mM Tris HCl pH 8.0, 10 mM EDTA and 10 µg/ml of RNase A), and incubated at 56°C for 1 h. After cooling, the various suspensions of DNA are extracted with 1 ml of phenol/chloroform as described above, and the chromosomal DNAs are precipitated with ethanol. The DNAs are resuspended in an Eppendorf tube containing a quantity of TE buffer such that the final quantity of DNA for each strain isolated is of the order of 250 µg/ml.

An aliquot of 1 µl of DNA of each strain isolated is amplified by PCR with the primers having the respective nucleotide sequences SEQ ID NO: 2 and SEQ ID NO: 3 (see sequence listing below), for 30 cycles (95°C/30 sec, 40°C/30 sec and 72°C/2 min) using *Pwo* polymerase from Boehringer. The PCR products are purified with the aid of the QIAGEN QIAquick kit, and the products are eluted in 50 µl of TE buffer. A sample of 20 µl of each product is digested with the restriction enzymes *Bam*HI and *Sal*I, and the 1.6 kb fragments are separated on an agarose gel (1%), they are purified with the aid of the QIAGEN QIAquick kit, they are then cloned into the *E. coli* vector pK19 (R.D. Pridmore, Gene 56, 309-312, 1987) previously digested



with *Bam*HI and *Sal*I and dephosphorylated, and the competent cells *E. coli* strain BZ234 (University of Basel collection, Switzerland) are transformed with each ligation product. The transformants are selected at 37°C on LB medium comprising 50 µg/ml of kanamycin, 30 ng/ml of X-gal and 10 ng/ml of IPTG. The white colonies containing the insert are cultured for 10 h on LB medium comprising 50 µl/ml of kanamycin, and the plasmid DNAs are isolated with the aid of the QIAGEN QIAprep8 kit.

Four µl of sample of each plasmid (1 pmol/µl: obtained from each strain isolated) are mixed with 4 µl of labelled primers IRD-41 (sequencing primers: MWG Biotech) and 17 µl of H_2O. For each strain isolated, 4 aliquots of 6 µl are dispensed to 4 wells of 200 µl, and 2 µl of a reaction mixture (Amersham; RPN2536) are added to them. The mixtures are amplified by PCR in the Hybaid Omn-E system by 1 cycle of 95°C for 2 min followed by 25 cycles of 95°C/30 sec, 50°C/30 sec and 72°C/1 min. The reaction products are then conventionally separated on a polyacrylamide gel, and the DNA sequence is read for each strain isolated. The DNA fragments thus sequenced represent the genomic part of the 16S ribosomal RNA.

The results show that all the strains isolated contain a nucleotide sequence similar, or even identical, to the sequence SEQ ID NO: 1 which is disclosed in the sequence listing below. These sequences exhibit numerous homologies with the 16S RNA sequences found in the species of lactic acid bacteria belonging to the genus *Streptococcus*, which leads to these strains being classified in the genus *Streptococcus*.

4. Identification by SDS-PAGE electrophoresis gel

The tests were carried out in accordance with the instructions provided by Pot *et al.* presented during a "workshop" organized by the European Union, at the University of Ghent, in Belgium, on 12 to 16

September 1994 (Fingerprinting techniques for classification and identification of bacteria, SDS-PAGE of whole cell protein).

In short, to cultivate the lactic acid bacteria, 10 ml of MRS medium (of Man, Rogosa and Sharpe) are inoculated with an MRS preculture of each strain of the new species of lactic acid baterium, as well as of each reference strain covering as many species of *Streptococcus* as possible. The media are incubated for 24 h at 28°C, they are plated on a Petri dish comprising a fresh MRS-agar medium, and the dishes are incubated for 24 h at 28°C.

To prepare the extract containing the proteins of the bacteria, the MRS-agar medium is covered with a pH 7.3 buffer containing 0.008 M of $Na_2HPO_4.12H_2O$, 0.002 M of $Na_2HPO_4.2H_2O$ and 8% NaCl. The bacteria are recovered by scraping the surface of the gelled medium, the suspension is filtered through a nylon gauze, it is centrifuged for 10 min at 9000 rpm with a GSA rotor, the pellet is recovered and taken up in 1 ml of the preceding buffer. The pellet is washed by repeating the centrifugation-washing procedure, finally about 50 mg of cells are recovered to which one volume of STB buffer pH 6.8 (per 1000 ml: 0.75 g Tris, 5 ml C_2H_6OS, 5 g of glycerol) is added, the cells are broken by ultrasound (Labsonic 2000), the cellular debris is centrifuged, and the supernatent containing the total protein is preserved.

An SDS-PAGE polyacrylamide gel 1.5 mm thick (Biorad-Protean or Hoefer SE600), crosslinked with 12% acrylamide in the case of the separating gel (12.6 cm in height) and 5% acrylamide in the case of the stacking gel (1.4 cm in height), is then conventionally prepared. For that, the polymerization of the two gel parts is carried out in particular in a thermostated bath at 19°C for 24 h and 1 h respectively, so as to reduce the gel imperfections as much as possible and to maximize the reproducibility of the tests.

The proteins of each extract are then separated on the SDS-PAGE electrophoresis gel. For that, 6 mA are applied for each plate containing 20 lanes until the dye reaches a distance of 9.5 cm from the top of the
5 separating gel. The proteins are then fixed in the gel, they are stained, the gel is dried on a cellophane, the gel is digitized by means of a densitometer (LKB Ultroscan Laser Densitometer, Sweden) linked to a computer, and the profiles are compared with each other
10 by means of the GelCompar® software, version 4.0, Applied Maths, Kortrijk, Belgium. Insofar as the tests were sufficiently standardized, the profiles of the various species of *Streptococcus* contained in a given library were also used during the digital comparison.

15 The results then show that all the strains tested belonging to the new species can be distinguished from all of the following species: *S. acidominimus*, *S. adjacens*, *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. bovis*, *S. canis*, *S.*
20 *caprinus*, *S. casseliflavus*, *S cecorum*, *S. constellatus*, *S. cremoris*, *S. cricetus*, *S. cristatus*, *S. defectivus*, *S. difficile*, *S. downei*, *S. dysgalactiae ssp. dysgalactiae*, *S. dysgalactiae ssp. equisimilis*, *S. equi*, *S. equi ssp. equi*, *S. equi ssp. zooepidemicus*, *S.*
25 *equinus*, *S. faecalis*, *S. faecium*, *S. ferus*, *S. gallinarum*, *S. gallolyticus*, *S. garvieae*, *S. gordonii*, *S. hansenii*, *S. hyointestinalis*, *S. hyovaginalis*, *S. iniae*, *S. intermedius*, *S. intestinalis*, *S. lactis*, *S. lactis cremoris*, *S. lactis diacetilactis*, *S. macacae*,
30 *S. mitis*, *S. morbillorum*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. parauberis*, *S. parvulus*, *S. phocae*, *S. plantarum*, *S. pleomorphus*, *S. pneumoniae*, *S. porcinus*, *S. pyogenes*, *S. raffinolactis*, *S. ratti*, *S. saccharolyticus*, *S. salivarius*, *S. sanguinis*, *S.*
35 *shiloi*, *S. sobrinus*, *S. suis*, *S. thermophilus*, *S. thoraltensis*, *S. uberis*, *S. vestibularis* and *S. viridans*.

All the results show that the degree of Pearson correlation between the strains deposited is at least

85. As a guide, Figure 1 represents a photograph of one of the electrophoresis gels, the filiation in the form of a tree, as well as the degree of Pearson correlation (indicated on the top left-hand scale). The strains LAB 1550, LAB 1551 and LAB 1553 refer specifically to the strains CNCM I-1921, I-1922 and I-1925. The strains LMG15061 and LAB 1607 were not deposited at the CNCM, but obviously form part of this new species.

In short, all the strains isolated clearly form part of a homogeneous group, which is distinct from the other species belonging to the genus *Streptococcus*.

Example 2 Mesophilic/thermophilic biotype

Some strains isolated in Example 1 represent a new particular biotype since they exhibit the remarkable property of being both mesophilic and thermophilic.

This property can be easily observed (1) by preparing, in parallel, several cultures of a mesophilic/thermophilic biotype in an M17-lactose medium at temperatures ranging from 20 to 50°C, (2) by measuring the absorbance values for the media at 540 nm after 16 h of culture, and (3) by grouping the results in the form of a graph representing the absorbance as a function of the temperature (graditherm).

Figure 1 represents the graditherm obtained with the strain CNCM I-1920. All the other strains isolated belonging to this particular biotype, in particular the strains CNCM I-1921 and I-1922, also give comparable graditherms.

Example 3 Texturing biotype

Several strains isolated in Example 1 have the remarkable property of being extremely texturing. This property can be observed with the aid of the rheological parameter of viscosity measured with a Bohlin VOR rotational rheometer (Bohlin GmbH, Germany).

For that, some of the strains isolated are cultured in a semi-skimmed milk at 38°C upto a pH of

5.2. In accordance with the manufacturer's instructions, a sample of each culture medium is then placed between a plate and a truncated cone of the same diameter (30 mm, angle of 5.4°, gap of 0.1 mm), then
5 the sample is subjected to a continuous rotating shear rate gradient which forces it to flow. The viscosity of the sample is then determined at a shear rate of 293 s^{-1}. The results of the rheology tests carried out with some of the strains isolated show that the culture
10 media thus fermented have a viscosity greater than 100 mPa.s, or even a viscosity exceeding 200 mPa.s in the case of the strains CNCM I-1922, I-1923, I-1924, I-1925 and I-1926.

For comparison, viscosities of the order of 54,
15 94, 104, 158 and 165 mPa.s are obtained, under the same operating conditions, with the strains *Lactobacillus helveticus* CNCM I-1449, *Streptococcus thermophilus* CNCM I-1351, *Streptococcus thermophilus* CNCM I-1879, *Streptococcus thermophilus* CNCM I-1590, *Lactobacillus*
20 *bulgaricus* CNCM I-800 and *Leuconostoc mesenteroides ssp. cremoris* CNCM I-1692, respectively, which are mentioned in patent applications EP699689, EP638642, EP97111379.0, EP750043, EP367918 and EP97201628.1, respectively (the strains CNCM I-800 and I-1692 are
25 reputed to be highly texturing strains).

Example 4 New exopolysaccharide

Some strains isolated in Example 1, belonging to the texturing biotype, in particular the strains
30 CNCM I-1923, I-1924, I-1925 and I-1926, produce an EPS of high molecular weight whose sugar composition is similar to those found in certain oligosaccharides in human breast milk. Analysis of the sugars constituting this polysaccharide is carried out in the following
35 manner.

The strains of the new species are cultured in 10% reconstituted skimmed milk, with shaking, for 24 h at 30°C, the pH being maintained at 5.5 by addition of a 2 N NaOH solution. The bacterial cells and the

proteins are removed from the culture medium by means of precipitation in an equal volume of a solution of 25% by weight of trichloroacetic acid, followed by centrifugation (10,000 g, 1 h). The EPSs are
5 precipitated by addition of an equivalent volume of acetone, followed by settling for 20 h at 4°C. The EPSs are recovered by centrifugation, the pellet is taken up in a 0.1 M NH_4HCO_3 solution pH 7, and the suspension is dialysed against water for 24 h. The insoluble
10 materials are then removed by ultracentrifugation, and the retentate containing the purified EPS is freeze-dried. The quantity of purified EPS, expressed as mg of glucose equivalent, is of the order of 40 mg per litre of culture.

15 The molecular weight of the EPS is determined by means of gel-filtration chromatography with the aid of a Superose-6 column connected to an FPLC system (Pharmacia), as described by Stingele *et al.*, J. Bacteriol., 178, 1680-1690, 1996. The results show that
20 all the strains CNCM I-1923, I-1924, I-1925 and I-1926 produce an EPS of a size greater than 2×10^6 Da.

100 mg glucose equivalent of the purified EPS are hydrolysed in 4 N TFA at 125°C for 1 h, before being derivatized and analysed by GLC chromatography
25 according to the method described by Neeser *et al.* (Anal. Biochem., 142, 58-67, 1984). The results show that the strains produce an EPS consisting of glucose, galactose and N-acetylglucosamine in a mean proportion of 3:2:1, respectively.

30

Example 5 Infant product

A whey, 18% hydrolysed with trypsine is prepared according to the recommendations of US 5,039,532, it is traditionally spray-dried in a stream
35 of hot air, and between 0.1 and 10% of the dry purified EPS described in Example 4 is incorporated into it. This product can be rapidly reconstituted in water. It is particularly suitable for a diet for children or breast-feeding infants because of its hypoallergenic

and tolerogenic properties to cow's milk, and because it is balanced from a carbohydrate composition point of view.

5 Example 6 Infant product

The dry purified EPS of Example 4 is hydrolysed in a 0.5 N trifluoroacetic acid (TFA) solution for 30-90 min and at 100°C, the TFA is evaporated off, the hydrolysate is suspended in water and the 10 oligosaccharides having 3 to 10 units of sugar (600 to 2000 Dalton) are separated by ultrafiltration.

A whey, 18% hydrolysed with trypsine is prepared according to the recommendations of US 5,039,532, it is traditionally spray-dried in a stream 15 of hot air, and between 0.1 and 10% of purified oligosaccharides described above is incorporated into it. This product can be rapidly reconstituted in water. It is particularly suitable for a diet for children or breast-feeding infants because of its hypoallergenic 20 and tolerogenic properties to cow's milk, and because it is balanced from a carbohydrate composition point of view.

Example 7 Pharmaceutical product

25 A pharmaceutical composition is prepared in the form of a capsule manufactured based on gelatin and water, and which contains 5 to 50 mg of the purified EPS of Example 4 or the purified oligosaccharides of Example 6.

30

Example 8 Pharmaceutical product

Pastilles consisting of a culture of the freeze-dried strain CNCM I-1924 are prepared and then compressed with a suitable binding agent. These 35 pastilles are particularly recommended for restoring an intestinal flora of lactic acid bacteria and for satisfying a balanced diet in terms of essential complex carbohydrates.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: SOCIETE DES PRODUITS NESTLE

(B) STREET: Av NESTLE 55

(C) CITY: VEVEY

(D) STATE OR PROVINCE: VAUD

(E) COUNTRY: SWITZERLAND

10 (F) POSTAL CODE: 1500

(ii) TITLE OF INVENTION: New species of lactic acid bacterium

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC Compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

20

(1) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1522 base pairs

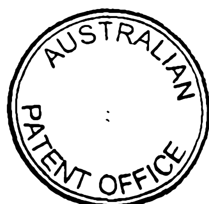
(B) TYPE: nucleotide

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 1



```
GTCGACAGAG TTCGATCCTG GCTCAGGACG AACGCTGGCG GCGTGCCTAA TACATGCAAG     60
TAGAACGCTG AAGACTTTAG CTTGCTAGAG TTGGAAGAGT TGCGAACGGG TGAGTAACGC    120
GTAGGTAACC TGCCTATTAG TGGGGGATAA CTATTGGAAA CGATAGCTAA TACCGCATAA    180
TAGTGTTTAA CACATGTTAG AGACTTAAAA GATGCAATTG CATCACTAGT AGATGGACCT    240
GCGTTGTATT AGCTAGTTGG TGGGGTAACG GCCTACCAAG GCGACGATAC ATAGCCGACC    300
TGAGAGGGTG ATCGGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC GGGAGGCAGC    360
AGTAGGGAAT CTTCGGCAAT GGGGGCAACC TGACCGAGCA ACGCCGCGTG AGTGAAGAAG    420
GTTTTCGGAT CGTAAAGCTC TGTTGTAAGA GAAGAACGTG TGTGAGAGTG GAAAGTTCAC    480
ACAGTGACGG TAACTTACCA GAAAGGGACG GCTAACTACG TGCCAGCAGC CGCGGTAATA    540
CGTAGGTCCC GAGCGTTGTC CGGATTTATT GGGCGTAAAG CGAGCGCAGG CGGTTTAATA    600
AGTCTGAAGT TAAAGGCAGT GGCTTAACCA TTGTTCGCTT TGGAAACTGT TAAACTTGAG    660
TGCAGAAGGG GAGAGTGGAA TTCCATGTGT AGCGGTGAAA TGCGTAGATA TATGGAGGAA    720
CACCGGTGGC GAAAGCGGCT CTCTGGTCTG TAACTGACGC TGAGGCTCGA AAGCGTGGGG    780
AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA CGATGAGTGC TAGGTGTTAG    840
GCCCTTTCCG GGGCTTAGTG CCGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGA    900
CCGCAAGGTT GAAACTCAAA GGAATTGACG GGGGCCGCAC AAGCGGTGGA GCATGTGGTT    960
TAATTCGAAG CAACGCGAAG AACTTACCAG GTCTTGACAT CCCGATGCTA TTTCTAGAGA   1020
TAGAAAGTTT CTTCGGAACA TCGGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG   1080
TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTCA   1140
GTTGGGCACT CTAGCGAGAC TGCCGGTGAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA   1200
TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG   1260
CAAGCCGGTG ACGGCAAGCA AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA   1320
ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC   1380
GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG   1440
GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAC AGATGATTGG GGTGAAGTCG   1500
TAACAAGGTA ACCGTAGGAT CC                                           1522
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 34 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

10 (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 2:

ATATCCGTTT TTTCGACAGA GTTYGATYCT GGCT

15 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

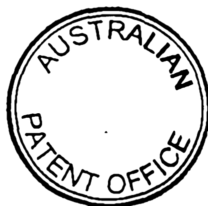
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 3:

25 ATATCCGGAT CCTACGGYTA CCTTGTTACG ACT



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

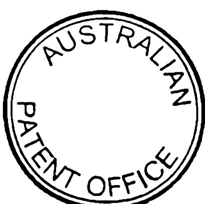
1. Strain of lactic acid bacterium,

- whose 16S ribosomal RNA is characteristic of the genus *Streptococcus*,

- whose total protein profile, obtained after culture of the bacterium in an MRS medium for 24 h at 28°C, extraction of the total proteins and migration of the proteins on an SDS-PAGE electrophoresis gel, exhibits a degree of Pearson correlation of at least 78 with respect to the profile obtained under identical conditions with the strain of lactic acid bacterium CNCM I-1920, but distinct from those of the recognized species belonging to the genus *Streptococcus,* namely *S. acidominimus, S. agalactiae, S. alactolyticus, S. anginosus, S. bovis, S. canis, S. caprinus, S. constellatus, S. cricetus, S. cristatus, S. difficile, S. downei, S. dysgalactiae, ssp. dysgalactiae, S. dysgalactiae ssp. equisimilis, S. equi, S. equi ssp, equi, S. equi ssp. zooepidemicus, S. equinus, S. ferus, S. gallolyticus, S, gordonii, S. hyointestinalis, S. hyovaginalis, S. iniae, S. intermedius, S. intestinalis, S. macacae, S. mitis, S. mutans, S. oralis, S. parasanguinis, S. parauberis, S. phocae, S. pleomorphus, S. pneumoniae, S. porcinus, S. pyogenes, S. ratti, S. salivarius, S. sanguinis, S. shiloi, S. sobrinus, S. suis, S. thermophilus, S. thoraltensis, S. uberis, S. vestibularis, S. viridans.*

2. Strain of lactic acid bacterium according to Claim 1, capable of fermenting D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-(D)-glucosamine, salicin, cellobiose, maltose, lactose, sucrose and raffinose.

3. Strain of lactic acid bacterium according to Claim 1 or Claim 2, chosen from the strains CNCM I-1920, I-1921, I-1922, I-1923, I-1924, I-1925 and I-1926.



4. Strain of lactic acid bacterium according to any one of Claims 1 to 3, wherein it has a growth optimum from about 28°C up to about 45°C.

5. Strain of lactic acid bacterium according to any one of Claims 1 to 4, which, when it ferments a semi-skimmed milk at 38°C up to pH 5.2, gives to the medium a viscosity which is greater than 100 mPa.s at a shear rate of the order of 293 s^{-1}.

6. Strain of lactic acid bacterium according to any one of Claims 1 to 5, wherein it produces an exopolysaccharide consisting of a succession of glucose, galactose and N-acetylglucosamine in a proportion of 3:2:1 respectively.

7. Use of a strain of lactic acid bacterium according to any one of Claims 1 to 6, for the preparation of a dietary composition, in particular an acidified milk.

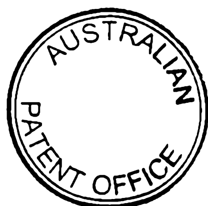
8. Use of a polysaccharide, capable of being secreted by a strain of lactic acid bacterium according to Claim 6, for the preparation of a dietary composition.

9. Use according to Claim 8, for the preparation of a composition intended to satisfy a balanced infant diet.

10. Dietary or pharmaceutical composition comprising a strain of lactic acid bacterium according to any one of Claims 1 to 6.

11. Dietary or pharmaceutical composition comprising a polysaccharide, capable of being secreted by the strain CNCM I-1924, consisting exclusively of a succession of glucose, galactose and N-acetylglucosamine in a respective proportion of 3:2:1.

12. Composition according to Claim 11, wherein it is an infant composition.



13. Composition according to Claim 12, wherein it is a hypoallergenic infant composition.

14. Composition according to any one of Claims 11 to 13, wherein the polysaccharide is in hydrolysed form, predominantly consisting of oligosaccharides having from 3 to 10
5 units of sugar.

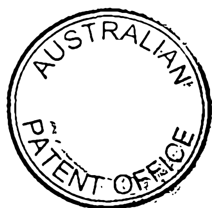
15. Polysaccharide, capable of being secreted by the strain CNCM I-1924, consisting exclusively of a succession of glucose, galactose and N-acetylglucosamine in a respective proportion of 3:2:1.

16. Strain of lactic acid bacterium, whose 16S ribosomal RNA is characteristic of the
10 genus *Streptococcus*, substantially as herein described with reference to any one of the examples but excluding comparative examples.

17. Use of a strain of lactic acid bacterium, whose 16S ribosomal RNA is characteristic of the genus *Streptococcus*, substantially as herein described with reference to any one of the examples but excluding comparative examples.

15 18. Use of a polysaccharide, capable of being secreted by a strain of lactic acid bacterium, whose 16S ribosomal RNA is characteristic of the genus *Streptococcus*, substantially as herein described with reference to any one of the examples but excluding comparative examples.

19. Dietary or pharmaceutical composition comprising a strain of lactic acid
20 bacterium, whose 16S ribosomal RNA is characteristic of the genus *Streptococcus*, substantially as herein described with reference to any one of the examples but excluding comparative examples.



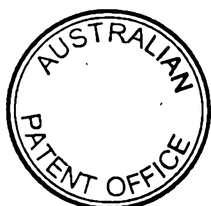
20. Dietary or pharmaceutical composition comprising a polysaccharide, capable of being secreted by the strain CNCM I-1924, substantially as herein described with reference to any one of the examples but excluding comparative examples.

21. Polysaccharide, capable of being secreted by the strain CNCM I-1924, substantially as herein described with reference to any one of the examples but excluding comparative examples.

DATED this 18th day of April 2001

SOCIETE DES PRODUITS NESTLE S.A.

Attorney: IVAN A. RAJKOVIC
Fellow Institute of Patent and Trade Mark Attorneys of Australia
of BALDWIN SHELSTON WATERS



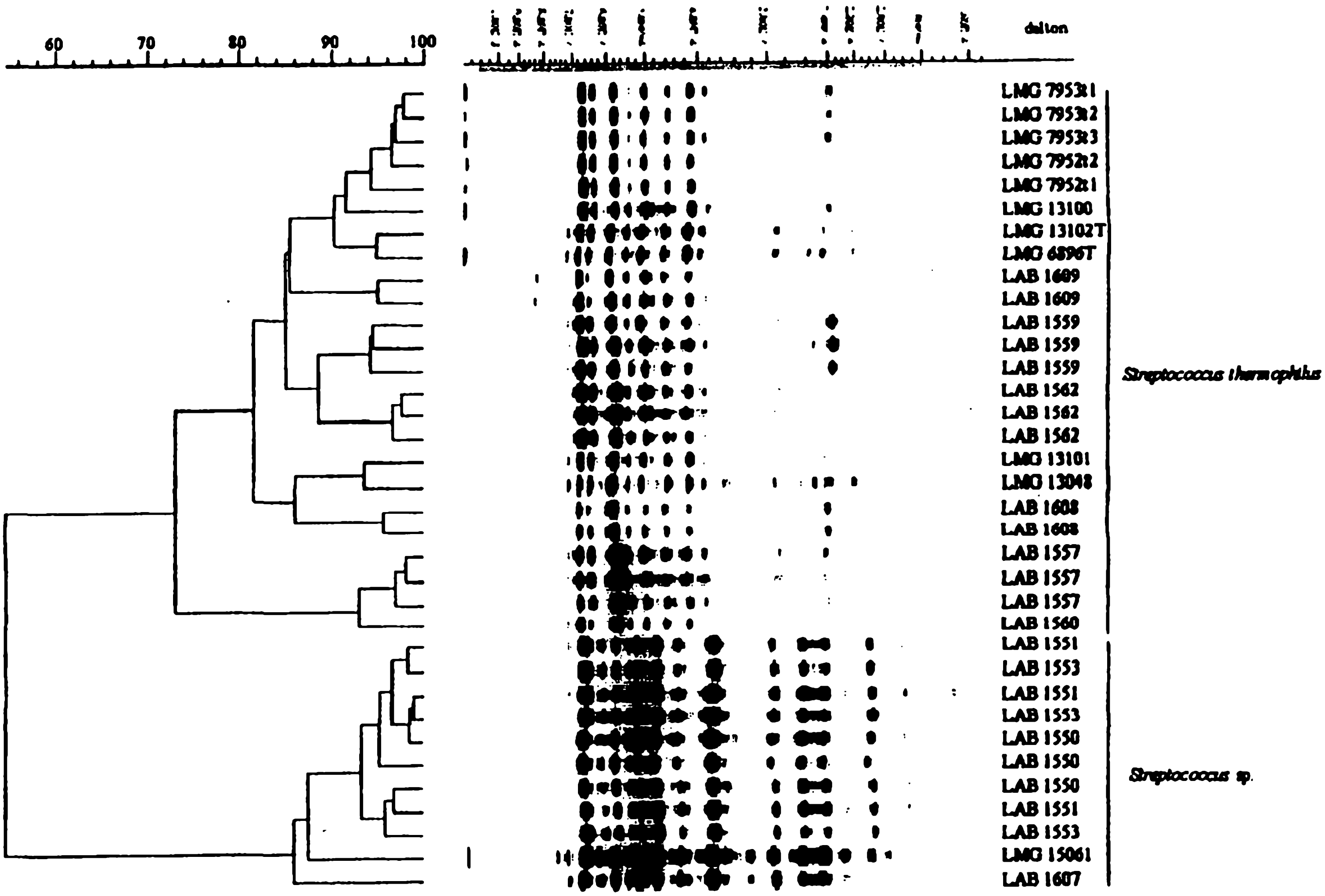


Figure 1

Figure 2