Title: Streptococcus thermophilus strains for treating Helicobacter pylori infection

Abstract: The present invention relates to a strain of Streptococcus thermophilus or a cell fraction thereof for use in the treatment or prevention of Helicobacter pylori infection.
STREPTOCOCCUS THERMOPHILUS STRAINS FOR TREATING HELICOBACTER PYLORI INFECTION

The present invention relates to the field of probiotics. Particularly, the invention pertains to the use of a strain of Streptococcus thermophilus for the treatment or the prevention of Helicobacter pylori infection.

According to a definition recently approved by the National Yogurt Association (NYA) or the International Life Science Institute (ILSI) in the USA, probiotics are living micro-organisms which upon ingestion in a sufficient amount exert health benefits beyond basic nutrition. Probiotic bacteria have been described among species belonging to the genera Lactobacillus, Bifidobacterium, Streptococcus and Lactococcus, commonly used in the dairy industry. Probiotics are thought to intervene at the level of the gut microbiota by impeding the development of pathogenic microorganisms and/or by acting more directly on the immune system.

Helicobacter pylori (H. pylori) is a Gram-negative spiral-shaped bacteria that colonizes the human gastric mucus layer of more than 50% of the world's population. While the majority of individuals infected with H. pylori is asymptomatic although their gastric epithelium show sign of inflammation, 15% to 20% of H. pylori infected individuals develop diseases. Indeed, H. pylori is the major causative agent of chronic active gastritis, peptic ulcer diseases, atrophy, metaplasia, dysplasia, gastric cancer and gastric mucosa associated lymphoid tissue (MALT) lymphoma (see for review Fox and Wang, 2007 and Polk and Peek, 2010).

During infection, H. pylori binds specifically to gastric epithelial cells lining the gastric epithelium through several adhesion molecules (adhesins) produced by the bacteria, such as BabA and SabA proteins. Adhesion to the gastric epithelial cells protects the bacteria from liquid flow, peristaltic movement and shedding of the mucous layer. H. pylori adhesion to the gastric mucosa induces signal transduction pathways within the gastric epithelial cells, leading to gastric epithelial cell damages and atrophy via oxidative stress, apoptosis and/or autophagy mechanisms. Accordingly, H. pylori adhesion to gastric epithelial cells is a key step in the establishment of an infection of the gastric mucosa.

The standard treatment in patients infected with H. pylori is two antibiotics associated to a proton pump inhibitor (PPI), so called triple therapy. However, H. pylori eradication rate following triple therapy is dropping down because of antibiotic resistance.
or poor compliance. Further, despite several clinical trials, there is no effective vaccine available on the market yet.

It appears from the foregoing that there is a need for alternatives or complements to triple therapy for the treatment or for the prevention of *H. pylori* infection.

The use of probiotics has been proposed as alternatives or complements to triple therapy for treating or preventing *H. pylori* infection. For instance, *Lactobacillus reuteri* is considered as a candidate probiotic for inhibiting the growth of *H. pylori* since it produces the potent antimicrobial substance reuterin (3-hydroxypropionaldehyde) (International Application WO 2004/031368). Boyanova *et al.* (2009) have found several *Lactobacillus delbrueckii subsp. bulgaricus* strains that inhibit the growth of *H. pylori* strains in vitro. Simova *et al.* (2009) disclose a *Lactobacillus delbrueckii* strain (BB18) producing an inhibitory peptide (bacteriocin) and strongly inhibiting *H. pylori*. Linsalata *et al.* (2004) found that the *Lactobacillus brevis* strain CD2 is capable of reducing the intragastric *H. pylori* load, and suggested that it might be due to the elevated arginine deiminase activity of this strain, which would deprive *H. pylori* of arginine, and inhibit their growth and proliferation.

The inventors have found that the bacterial species *Streptococcus thermophilus* (*S. thermophilics*) is capable of decreasing the load of *H. pylori* strains in vitro.

Accordingly, a subject of the present invention is a *Streptococcus thermophilus* strain for use for treating or preventing *Helicobacter pylori* infection.

Said *Streptococcus thermophilus* can be used as a medicament, including a pharmaceutical composition and a functional food.

Said *S. thermophilus* strain is capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*.

In a preferred embodiment, said *S. thermophilus* strain is the strain CNCM 1-1520. This strain was deposited by the Applicant, according to the Budapest Treaty, at CNCM (Collection Nationale de Cultures de Microorganismes, 25 rue du Docteur Roux, Paris) on December 30, 1994. This strain is disclosed in International Application WO 96/20607. This strain is also referred to as DN-001 147.

The present invention also encompasses mutant strains or genetically transformed strains derived from the parent strain CNCM 1-1520, provided that they are capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*. Method for assessing the capacity of a *S. thermophilus* strain to decrease the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori* are described in the
Examples below. These mutant or genetically transformed strains can be strains wherein one or more endogenous gene(s) of the parent strain CNCM 1-1520 has (have) been mutated, for instance to modify some of their metabolic properties (e.g., their ability to ferment sugars, their resistance to acidity, their survival to transport in the gastrointestinal tract, their post-acidification properties or their metabolite production). They can also be strains resulting from the genetic transformation of the parent strain CNCM 1-1520 by one or more gene(s) of interest, for instance in order to give to said genetically transformed strains additional physiological features, or to allow them to express proteins of therapeutic or vaccinal interest that one wishes to administer through said strains. These mutant or genetically transformed strains can be obtained from the parent strain CNCM 1-1520 strain by means of the conventional techniques for random or site-directed mutagenesis and genetic transformation of *Streptococcus*, such as those described by Biswas *et al.*, 1993 and Maguin *et al.*, 1996, or by means of the technique known as "genome shuffling", such as described by Yu *et al.*, 2008.

A subject of the present invention is also a cell fraction which can be obtained from a *S. thermophilus* strain capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*, preferably the strain CNCM 1-1520, provided that said cell fraction is capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*, for use for treating or preventing *H. pylori* infection. Said cell fraction is in particular DNA preparations or bacterial wall preparations obtained from cultures of said strain. It may also be culture supernatants or fractions of these strains. The cell fractions suitable for this use can be chosen, for example, by testing their properties on the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*.

A subject of the present invention is also a composition comprising a *Streptococcus thermophilus* strain according to the present invention, preferably the strain CNCM 1-1520, or a cell fraction according to the present invention, for use for treating or preventing *H. pylori* infection.

In the composition of the invention, said strain can be used in the form of whole bacteria which may be living or dead. Alternatively, said strain can be used in the form of a bacterial lysate. Preferably the bacterial cells are present as living, viable cells.

The composition of the invention can be in any form suitable for administration, in particular oral administration. This includes for instance solids, semi-solids, liquids, and
powders. Liquid composition are generally preferred for easier administration, for instance as drinks.

The composition can comprise at least $10^5$ cfu, preferably at least $10^6$ cfu, per gram dry weight, of at least one bacterial strain as mentioned above.

The composition can further comprise other strains of *Streptococcus thermophilus* and/or other strains of bacteria than the strains according to the present invention, in particular probiotic strain(s), such as *Lactobacillus, Bifidobacterium* and *Lactococcus* strain(s).

In a preferred embodiment, the composition comprises the *Streptococcus thermophilus* strain CNCM 1-1520, the *Streptococcus thermophilus* strain CNCM 1-1521 (also referred to as DN-001 339) and the *Lactobacillus bulgaricus* strain CNCM 1-1519 (also referred to as DN-100 182), and optionally a *Lactobacillus paracasei* strain, preferably the *Lactobacillus paracasei* subsp. *paracasei* CMCM 1-1518 (also referred to as DN-1 14 001). All these strains are described in International Application WO 96/20607.

When the bacteria are in the form of living bacteria, the composition may typically comprise $10^5$ to $10^{13}$ colony forming units (cfu), preferably at least $10^6$ cfu, more preferably at least $10^7$ cfu, still more preferably at least $10^8$ cfu, and most preferably at least $10^9$ cfu per gram dry weight of the composition. In the case of a liquid composition, this corresponds generally to $10^4$ to $10^{12}$ colony forming units (cfu), preferably at least $10^5$ cfu, more preferably at least $10^6$ cfu, still more preferably at least $10^7$ cfu, and most preferably at least $10^9$ cfu/ml.

The composition can be a pharmaceutical composition or a nutritional composition, including food products, food supplements and functional food. More particularly, the composition can be a medicament, including a pharmaceutical composition and a functional food.

A "food supplement" designates a product made from compounds usually used in foodstuffs, but which is in the form of tablets, powder, capsules, potion or any other form usually not associated with aliments, and which has beneficial effects for one's health. A "functional food" is an aliment which also has beneficial effects for one's health. In particular, food supplements and functional food can have a physiological effect - protective or curative - against a disease, for example against a chronic disease.

The nutritional composition according to the invention also includes a baby food, an infant milk formula or an infant follow-on formula. Preferably the present composition is a nutraceutical or a pharmaceutical product, a nutritional supplement or medical food.
The composition can be a dairy product, preferably a fermented dairy product. The fermented product can be present in the form of a liquid or present in the form of a dry powder obtained by drying the fermented liquid. Examples of dairy products include fermented milk and/or fermented whey in set, stirred or drinkable form, cheese and yoghurt.

The fermented product can also be a fermented vegetable, such as fermented soy, cereals and/or fruits in set, stirred or drinkable forms.

In a preferred embodiment, the fermented product is a fresh product. A fresh product, which has not undergone severe heat treatment steps, has the advantage that the bacterial strains present are in the living form.

A subject of the present invention is also the use of a *S. thermophilics* strain as defined above, preferably the strain CNCM 1-1520, or a composition as defined above for the manufacture of a medicament for treating or preventing *H. pylori* infection.

A subject of the present invention is also a method for treating or preventing *H. pylori* infection in a subject in need thereof, said method comprising administering to said subject a therapeutically effective amount of a *S. thermophilics* strain as defined above, preferably the strain CNCM 1-1520, or a composition as defined above.

Determination of a therapeutically effective amount is well known from the person skilled in the art, especially in view of the detailed disclosure provided herein.

A subject of the present invention is also a method for the manufacture of a medicament for treating or preventing *H. pylori* infection, said method comprising incorporating a *S. thermophilus* strain as defined above, preferably the strain CNCM 1-1520, or a cell fraction as defined above, into at least one pharmaceutically acceptable diluent, carrier or excipient.

As used herein, the treatment or prevention encompasses inter alia: preventive infection and/or decreasing the load of *H. pylori*. The treatment or prevention also encompasses addressing at least one of the symptoms associated with *H. pylori* mentioned below.

Methods for diagnosing a *H. pylori* infection are known in the art. By way of example, diagnosis of a *H. pylori* infection can be made by checking by a blood antibody test, a stool antigen test or the carbon urea breath test. It can also be made by biopsy under endoscopy followed by an urease test, a histological examination, a microbial culture or a quantitative Real-Time PCR.
The symptoms or diseases associated with *H. pylori* infection are stomach ache, abdominal pain, regurgitation, vomiting, belching, flatulence, nausea, chronic active gastritis, peptic ulcer diseases, atrophy, metaplasia, dysplasia, gastric cancer and gastric mucosa associated lymphoid tissue (MALT) lymphoma.

The present invention will be understood more clearly from the further description which follows, which refers to examples illustrating the capacity of the *S. thermophilus* strain CNCM 1-1520 of decreasing the load of *H. pylori* strains in vivo, as well as to the appended figures.

**Figure 1** shows the change in the weight (in grams) of non infected mice, mice infected with *H. pylori* SSI receiving a control product, or infected with *H. pylori* SSI and treated with *S. thermophilus* strain CNCM 1-1520, measured just before the treatment (first bar), 3 weeks after the treatment (second bar) and just before sacrifice (third bar), obtained for 2 independent experiments.

**Figure 2** shows the score of infection obtained by immunohistochemistry using anti-*H. pylori* antibodies in mice (i) non-infected with *H. pylori*, (ii) infected with *H. pylori* SSI receiving a control product (non-fermented milk) and (iii) infected with *H. pylori* SSI and treated with *S. thermophilus* CNCM 1-1520. Definition of scores: 0: no infected gland, 1: rare infected glands, 2: 25% infected glands, 3: from 25 to 50% infected glands, 4: > 50 % infected glands.

**Figure 3** shows the quantification of *H. pylori* SSI DNA obtained by Real-Time PCR in mice (i) non-infected with *H. pylori*, (ii) infected with *H. pylori* SSI but receiving a control product (non-fermented milk) and (iii) infected with *H. pylori* SSI and treated with *S. thermophilus* CNCM 1-1520.

**Figure 4** shows the change in the weight (in grams) of non infected mice, mice infected with *H. pylori* SSI receiving a control product, or infected with *H. pylori* SSI and treated with *S. thermophilus* strain CNCM 1-1520, measured just before the treatment (first bar), 3 weeks after the treatment (second bar) and just before sacrifice (third bar), obtained for 2 independent experiments.

**Figure 5** shows the quantification of *H. pylori* SSI obtained by bacterial culture on plate (in cfu (Colony Forming Unit) per gram of mice stomach) (i) non-infected with *H. pylori*, (ii) infected with *H. pylori* SSI but receiving a control product (non-fermented milk) and (iii) infected with *H. pylori* SSI and treated with *S. thermophilus* CNCM 1-1520.
EXAMPLE 1: EFFECT OF THE S. THERMOPHILUS STRAIN CNCM 1-1520 ON THE LOAD OF H. PYLORI IN A MICE MODEL DETERMINED BY HISTOLOGICAL AND qRT-PCR METHODS

1.1 Material & Methods

*Helicobacter pylori*

*H. pylori* strain SSI having a very good colonization ability of mouse gastric mucosa (Lee et al., 1997) was used. Identity of the strain was checked by sequencing the genes *glm, hspA* and *vacA* (Raymond et al., 2004; Espinoza et al., 2011; Zhang et al., 2007).

*Streptococcus thermophilics*

Milk product fermented by *S. thermophilic* strain CNCM 1-1520 was prepared as follows: First culture in M17 was prepared from frozen strain and incubated at 37°C for 17h. A second culture was prepared in skimmed milk enriched with yeast extract (2g/L) by inoculation at 1% from the first culture and incubated at 37°C for 17h. A third culture was prepared in milk enriched with yeast extract (2g/L) by inoculation at 1% from the second culture and incubation at 37°C until pH 4.7 was reached. The product was finally prepared by inoculation of milk enriched with yeast extract (2g/L) at 1% with the third culture until pH 4.8 was reached. Products were stored at -80°C. Bacterial count was carried out in M17 after 48h incubation. Bacterial count was 1.5x10^9 cfu/mL.

*Mice*

40 BALB/cBy/J female mice of 5 weeks old (Charles River, France) and tested as SPF (« specific pathogen free ») were split into groups: 2 groups of 15 mice were infected and 1 group of 10 mice was used as non infected control. Mice were fed with food poor in vitamins to enhance the lesion development induced by *H. pylori*.

*Infection (8 weeks)*

6 weeks old mice received a hydric diet for 1 day and then were force-fed the following morning with 250 μL of an enriched suspension of the strain *H. pylori* SSI (1 to 2 Petri dishes of *H. pylori* for 5 mice). The mice were put in a cage with a normal diet. Then, the mice received a hydric diet again in the evening. This protocol was repeated for 3 days.

*Treatment (6 weeks)*

Eight weeks after their infection, mice were treated for 6 weeks with milk products containing *S. thermophilic* CNCM 1-1520. 120 g of milk product were given per cage per day in feeding-bottles instead of water. The feeding-bottles were changed every
day. To assess the quantity of products ingested per animal, the feeding-bottles were weighed. Further, mice were weighted just before the treatment, 3 weeks after the treatment and just before sacrifice (results are shown in Figure 1).

Mice control groups received milk enriched with yeast extract (2g/L) (i.e., without any *S. thermophilus* strain).

**Sacrifices**

Mice were sacrificed by cervical dislocation. Laparotomy was performed. Stomachs were isolated and gastric mucosa was washed in physiological serum.

Stomach was cut through the middle from the esophagus to the duodenum. For the right half stomach, cardia was eliminated, and then this half stomach was put in physiological serum to be used for the molecular study. The left half stomach was used for histology.

**Histology**

The left half stomach was fixed 1 night in 3.7% formol and washed with 70% ethanol and then paraffin-embedded and sectioned at 3 μm thickness.

Immunohistochemistry was carried out with an antibody anti-*H. pylori* antigens: primary antibody: anti-*H. pylori* (Dako, Ref. B0471); secondary antibody and DAB: Dako EnVision+ System-HRP (DAB) (Dako, Ref. K401 1).

**Molecular study fq RT-PCR**

Right stomachs were homogenized (disrupted) in 0.2 ml physiological serum with a Potter-Elvehjem (the tube was weighted with and without the stomach tissue to know the weight of the tissue).

Total DNA was extracted from the crushed stomach with Arrow Stool DNA kit (NorDiag, Norway) following supplier recommendations. For each crushed stomach total DNA was resuspended in 180 μL TRIS buffer (10 mM).

Presence of DNA of *H. pylori* was quantified in DNA extracts by Real-Time PCR. Amplification was done with primers targeting 23S rRNA gene, present in two copies in *H. pylori* following the method described by Oleastro *et al.* (2003). For 20μl of mix (MgCl₂ 25mM, primers HPY-A et HPY-S 20μM described by Menard *et al.*, 2002, sensor probe that is 5’ labeled with LC-Red 640 and 3’ phosphorylated and anchor probe that is 3’ labeled with fluorescein (both probes described by Oleastro *et al.* 2003) 20μM, buffer containing the enzyme (10X, kit FastStart DNA Master Hybridization Probes, Roche Diagnostics), 5μl DNA at 200 ng/μl was added to be amplified in Light Cycler ROCHE, using the following program:
Denaturation: 95°C 10min
Amplification: 50 cycles 20°C/sec
95°C 0 sec
60°C 20 s
72°C 12 sec
Fusion: 95°C 0 sec
38°C 50 sec 20°C/sec

1.2 Results
The scores of infection obtained by immunohistochemistry are shown in Figure 2. These results show that administration of a milk product fermented with the *S. thermophilus* strain CNCM 1-1520 to mice infected with *H. pylori* decreases (not significantly) the score of infection compared with the score obtained with the treatment with the milk control.

The results obtained by Real-Time PCR are shown in Figure 3. These results show that, in mice, the treatment with the milk product fermented with the *S. thermophilus* strain CNCM 1-1520 significantly decreases the load of *H. pylori* compared to the treatment with the milk control.

**EXAMPLE 2: EFFECT OF THE *S. THERMOPHILUS* STRAIN CNCM 1-1520 ON THE LOAD OF *H. PYLORI* IN A MICE MODEL DETERMINED BY MICROBIOLOGICAL METHOD**

2.1 Material & Methods
The material & methods for this experiment are the same as those described in Example 1 above regarding the *H. pylori* strain, the *S. thermophilus* strain CNCM 1-1520, the mice, the infection, treatment and sacrifice of the mice, with the following exceptions:
- the bacterial count of *S. thermophilus* strain CNCM 1-1520 was 1.43x10^9 cfu/mL;
- the change in the weight of the treated mice is shown in Figure 4;
- only the right half stomach of the mice was used for the microbiology study.

**Microbiology study: culture of *H. pylori***

The half stomachs were browsed in 0.2ml physiological serum with a Potter (tube is weighed with the liquid with and without stomach to deduce the exact weigh of tissue), 100μl of dilutions (10^{-1} to 10^{-4}) were spread on Petri dish containing pylori medium GSSA (Glaxo Selective Supplement A (20 μg/ml bacitracin, 1.07 μg/ml nalidixic acid, 0.33 μg/ml polymyxin B, and 10μg /ml vancomycin) enriched with 10% blood. Bacterial
count was earned out at 37°C after 5 to 7 days incubation under microaerobic condition. *H. pylori* was identified by phenotypic and biochemistry behaviors (morphology, urease and oxydase assays).

### 2.2 Results

The results obtained by microbiology for the *S. thermophilus* strain CNCM 1-1520 are shown in Figure 5. These results show that, in mice, the treatment with the milk product fermented with the strain CNCM 1-1520 significantly decreases the load of *H. pylori* compared to the treatment with the milk control.

### REFERENCES

CLAIMS

1. A *Streptococcus thermophilus* strain for use for treating or preventing *Helicobacter pylori* infection.

2. A *S. thermophilus* strain according to claim 1, characterized in that said strain is capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*.

3. A *S. thermophilus* strain according to claim 1 or claim 2, characterized in that it is the strain CNCM 1-1520.

4. A cell fraction obtained from a *S. thermophilus* strain as defined in any of claims 1 to 3, wherein it is capable of decreasing the load of *H. pylori* strains in the stomach of a subject infected with *H. pylori*, for use for treating or preventing *H. pylori* infection.

5. A composition comprising a *S. thermophilus* strain as defined in any of claims 1 to 3 or a cell fraction as defined in claim 4 for use for treating or preventing *H. pylori* infection.

6. The composition according to claim 5, characterized in that it comprises at least 10⁵ cfu, preferably at least 10⁶ cfu, per gram dry weight, of a *S. thermophilus* strain as defined in any of claims 1 to 3.

7. The composition according to claim 5 or claim 6, characterized in that it is a nutritional composition.

8. The composition according to claim 7, characterized in that it is a dairy product.
Figure 2

Figure 3
Figure 4
Figure 5
## INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV.** C12N1/20  A61K39/02  

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)  
C12N A61K

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

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>KR 2013 0002534 A (BKBIO co LTD [KR]; GACHON UNIVERSITY OF INDUSTRY ACADEMIC COOPERATION) 8 January 2013 (2013-01-08) page 3 - page 9</td>
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<td>A</td>
<td>WO 96/20607 A1 (GERVAIS DANONE co [FR]; BOULEY CHRISTINE [FR]; OUDOT ELISABETH [FR]; D) 11 July 1996 (1996-07-11) cited in the application on page 1 - page 14; claims 1-7</td>
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Further documents are listed in the continuation of Box C.  
See patent family annex.

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  * "A" document defining the general state of the art which is not considered to be of particular relevance
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**Date of the actual completion of the international search**  
5 June 2013

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14/06/2013

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