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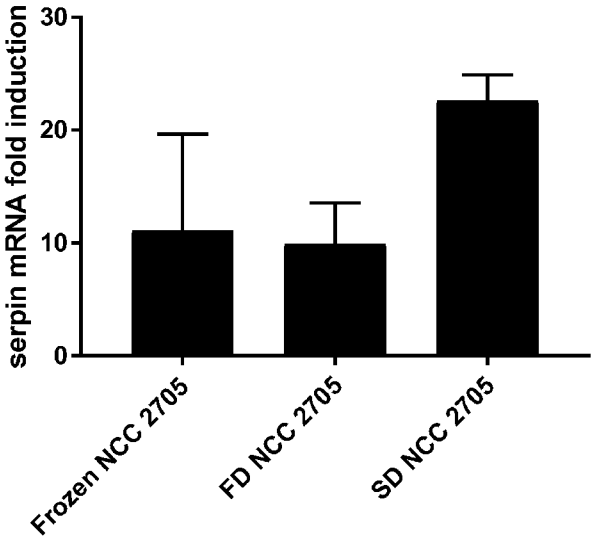


Figure 1

(57) Abstract: Use of spray drying for increasing serpin expression in *Bifidobacterium longum* strain CNCM I-2618.

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[Continued on next page]

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SERPIN PRODUCTION

FIELD OF THE INVENTION

The present invention relates to bacteria expressing serpin, methods for increasing serpin production in bacteria, and uses thereof.

BACKGROUND TO THE INVENTION

Gluten-related disorders comprise all diseases triggered by gluten. They include, amongst other pathophysiology, celiac disease and non-celiac gluten sensitivity. Currently, the incidence of a wide spectrum of gluten-related disorders is growing all around the world, especially for celiac disease and non-celiac gluten sensitivity. Both diseases are triggered by ingestion of gluten. Both innate and adaptive immunity are implicated in celiac disease while innate immunity is implicated in non-celiac gluten sensitivity.

A life-long gluten free diet is the gold standard treatment for celiac disease and non-celiac gluten sensitivity patients, although it may have some limitations on the extraintestinal manifestations of the disease (Sedghizadeh et al., 2002, Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 94(4), 474-478). It has been shown that following a strict gluten free diet is very difficult as low level cross-contaminations are difficult to avoid and may happen through the whole food production chain, from grains growth to manufacturing processing (Mitchison et al., 1991, Gut, 32(3), 260-265). Furthermore, it has been described that up to 3 g of hidden gluten might be consumed daily under a strict gluten free diet (Aziz et al., 2014, The American journal of gastroenterology, 109(9), 1498).

Celiac disease is prevalent especially in the United States and Europe where around 1 % of subjects had positive antibody tests (Dubé et al., 2005, Gastroenterology, 128(4), S57-S67). It is a complex disorder which arises from a complicated interaction among various immunologic, genetic, and environmental factors (Alaedini & Green, 2005). It is triggered by the digestion of wheat gluten and other related cereal proteins such as rye and barley proteins. Symptoms linked with celiac disease are growth retardation, irritability and pubertal delay in children, and many gastrointestinal symptoms such as discomfort, diarrhoea, occult stool, steatorrhea and flatulence, (Dubé et al., 2005; Sedghizadeh et al., 2002).

Non-celiac gluten sensitivity (also named non-celiac wheat sensitivity) is an emerging condition. It is defined as a clinical entity induced by the ingestion of gluten leading to intestinal and/or

extraintestinal symptoms which could be improved by removing the gluten-containing foodstuff from the diet (Lundin & Alaedini, 2012). In addition to gliadin (the main cytotoxic antigen of gluten), other proteins/peptides present in gluten and gluten-containing cereals (wheat, rye, barley, and their derivatives) may play a role in the development of symptoms. Non-celiac gluten sensitivity is the most common syndrome of gluten-related disorders with prevalence rates between 0.5–13 % in the general population (on average 5 %) (Catassi et al., 2013, Nutrients, 5(10), 3839-3853).

Serine protease inhibitors (serpin) are a superfamily of proteins found in eukaryotes (Gettins, 2002, Chemical reviews, 102(12), 4751-4804) and prokaryotes (Kantyka et al., Biochimie, 92(11), 1644-1656).

Recently, human serine protease inhibitors have been shown to play an important role in gluten-related disorders. Elafin is human serine protease inhibitor which shows potent inhibitory capacity against various forms of elastases and proteinase (Ying & Simon, 1993, Biochemistry, 32(7), 1866-1874). Elafin is expressed throughout the epithelium of the gastrointestinal tract and its expression and induction is decreased in patients with inflammatory bowel disease and celiac disease (Baranger, Zani, Labas, Dallet- Choisy, & Moreau, 2011; Motta et al., 2012). Recently, elafin has been identified as a substrate for the cross-linking activity of TG2 (Baranger et al., 2011, PloS one, 6(6), e20976; Motta et al., Science translational medicine, 4(158), 158ra144-158ra144). *In-vitro* data shows that the addition of elafin moderately inhibits transglutaminase 2 (TG2) thus inhibiting the deamidation of the digestion-resistant 33-mer gliadin peptide, which is one of the potential triggers of the adaptive immune response in CD (McCarville et al. 2015, Current opinion in pharmacology, 25, 7-12).

Delivery of elafin, produced by a recombinant *Lactococcus lactis* has been shown to reduce gluten-induced pathology and normalize intestine inflammation in a mouse model of gluten sensitivity (Galipeau et al., 2014, The American journal of gastroenterology, 109(5), 748-756). However, this proposed therapy is based on a genetically modified microorganism (GMO) and is therefore not compatible with a food application, as consumer acceptance of GMO is very low.

More recently, serpins have been reported in prokaryotes. *In silico* analysis revealed the presence of genes encoding serpin-like proteins in different *Bifidobacterium* species. The protein encoded by *B. longum subsp longum* (named *B. longum*) NCC 2705 displayed similar antiprotease activity to those of human serpin (Ivanov et al 2006, Journal of Biological Chemistry, 281(25), 17246-17252).

B. longum NCC 2705 was deposited with the Institute Pasteur according to the Budapest Treaty on 29th January 2001 receiving the deposit no. CNCM I-2618.

It has recently been shown that *B. longum* NCC 2705 (CNCM I-2618), through its serpin production can improve gluten induced pathophysiology in a mouse model of gluten sensitivity, showing its potential as solution for gluten related disorders (McCarville et al., 2017, Appl. Environ. Microbiol. Vol. 83, no. 19, e01323-17).

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that spray-drying preparations of *B. longum* strain CNCM I-2618 increases the level of serpin expression. *B. longum* strain CNCM I-2618 in the form of a spray-dried powder is associated with increased levels of serpin.

Thus, according to an aspect of the present invention, there is provided use of spray drying for increasing serpin expression in *B. longum* strain CNCM I-2618.

In an embodiment the *B. longum* strain CNCM I-2618 is cultured in a medium comprising the sugar selected from lactose, fructose and raffinose, prior to spray drying.

According to another aspect of the present invention there is provided *B. longum* strain CNCM I-2618 in the form of a spray dried powder.

According to another aspect of the present invention there is provided a process for increasing serpin concentration in a preparation of *B. longum* strain CNCM I-2618, wherein the process comprises spray drying *B. longum* strain CNCM I-2618.

In one embodiment, the process comprises growing *B. longum* strain CNCM I-2618 in a culture medium, followed by spray drying *B. longum* strain CNCM I-2618.

In one embodiment, the process comprises growing *B. longum* strain CNCM I-2618 in a culture medium comprising a sugar selected from lactose, fructose and raffinose, or combinations thereof. The present inventors have shown that these sugars enhance serpin expression in the *B. longum* strain CNCM I-2618.

In one embodiment, the culture medium comprises the sugar selected from lactose, fructose and raffinose at a concentration of 0.01 to 8 wt%, 0.02 to 6 wt%, 0.02 to 5 wt%, 0.02 to 4 wt%, 0.02

to 3 wt%, 0.02 to 2 wt %, 0.02 to 1 wt%, 0.02 to 0.5 wt %, 0.05 to 0.15 wt %, 0.08 to 0.12 wt %, or about 0.1 wt%.

According to another aspect of the present invention there is provided *B. longum* strain CNCM I-2618 in the form of a spray dried powder, wherein the spray dried powder is produced by growing *B. longum* strain CNCM I-2618 in a culture medium comprising a sugar selected from lactose, fructose and raffinose, followed by spray drying the *B. longum* strain CNCM I-2618.

According to another aspect of the present invention there is provided a composition comprising the *B. longum* strain CNCM I-2618 in the form of a spray dried powder of the invention.

The composition may be, for example, a food, a medical food, a tube feed, or a nutritional supplement.

In one embodiment the food is selected from milk, yoghurt, curd, cheese, fermented milks, milk based fermented products, rice based products, milk based powders, infant formulae and pet food.

In another embodiment, the composition is a pharmaceutical composition wherein the pharmaceutical composition comprises one or more pharmaceutically acceptable carriers, diluents and/or excipients.

According to another aspect of the present invention there is provided the spray dried powder or composition of the invention for use in the treatment or prevention of conditions related to gluten sensitivity or involving the reduced activity of serine protease inhibitors.

According to another aspect of the present invention there is provided the spray dried powder or composition of the invention for use in the treatment or prevention of a gluten-related disorder.

According to an aspect of the present invention there is provided the spray dried powder or composition of the invention for use in the treatment or prevention of celiac disease, non-celiac gluten sensitivity, gluten ataxia, dermatitis herpetiformis and wheat allergy.

According to another aspect of the present invention there is provided the spray dried powder or composition of the invention for use in the treatment or prevention of inflammatory bowel disease.

DESCRIPTION OF THE DRAWINGS

Figure 1: serpin mRNA fold induction in spray dried and freeze dried *B. longum* CNCM I-2618. Fold induction calculate based on standard lab growth conditions (MRSc). "Frozen NCC 2705": probiotic control, frozen biomass of CNCM I-2618 (no drying applied). "SD NCC 2705": *B. longum* CNCM I-2618 produced by spray-drying. "FD NCC 2705": *B. longum* CNCM I-2618 produced by freeze-drying.

Figure 2 – *In vivo* inhibition of elastase activity by spray dried and freeze dried *B. longum* CNCM I-2618 preparations in NOD/DQ8 gluten sensitive mice. All mice were sensitized and challenged with gluten. Control: no administration of probiotic. Frozen NCC 2705: probiotic control, frozen biomass of CNCM I-2618, (no drying applied). SD NCC 2705: *B. longum* CNCM I-2618 produced by spray-drying. FD NCC 2705: *B. longum* CNCM I-2618 produced by freeze-drying.

DETAILED DESCRIPTION OF THE INVENTION

Composition

The composition of the present invention may be in the form of a food, a medical food, a tube feed, a nutritional composition, or a nutritional supplement. The term "nutritional supplement" refers to a product which is intended to supplement the general diet of a subject.

In one embodiment, the food is selected from milk, yogurt, curd, cheese, fermented milks, milk-based fermented products, rice based products, milk based powders, infant formulae and pet food.

The composition may be in the form of a medical food. The term "medical food" as used herein refers to a food product specifically formulated for the dietary management of a medical disease

or condition. The medical food may be administered under medical supervision. The medical food may be for oral ingestion or tube feeding.

The composition may be in the form of a tube feed. The term “tube feed” refers to a product which is intended for introducing nutrients directly into the gastrointestinal tract of a subject by a feeding tube. A tube feed may be administered by, for example, a feeding tube placed through the nose of a subject (such as nasogastric, nasoduodenal, and nasojejunal tubes), or a feeding tube placed directly into the abdomen of a subject (such as gastrostomy, gastrojejunostomy, or jejunostomy feeding tube).

The composition may in the form of a pharmaceutical composition and may comprise one or more suitable pharmaceutically acceptable carriers, diluents and/or excipients.

Examples of such suitable excipients for compositions described herein may be found in the “Handbook of Pharmaceutical Excipients”, 2nd Edition, (1994), Edited by A Wade and PJ Weller. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985).

Examples of suitable carriers include lactose, starch, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents include ethanol, glycerol and water.

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s) and/or solubilising agent(s). Examples of suitable binders include starch, gelatin, natural sugars such as glucose, anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and polyethylene glycol.
Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like.

Preservatives, stabilisers, dyes and even flavouring agents may be provided in the composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

Nutritionally acceptable carriers, diluents and excipients include those suitable for human or animal consumption and that are used as standard in the food industry. Typical nutritionally acceptable carriers, diluents and excipients will be familiar to the skilled person in the art.

The composition may be in the form of a tablet, dragee, lozenge, capsule, gel cap, powder, granule, solution, emulsion, suspension, coated particle, spray-dried particle or pill.

In an alternative embodiment the composition may be in the form of a composition for topical administration, such as a gel, cream, ointment, emulsion, suspension or solution for topical administration.

It is clear to those skilled in the art that an ideal dose will depend on the subject to be treated, its health condition, sex, age, or weight, for example, and the route of administration. The dose to be ideally used will consequently vary but can be determined easily by those of skill in the art.

However, generally, it is preferred if the composition of the present invention comprises between 10^6 and 10^{10} cfu and/or between 10^6 and 10^{10} cells of *B. longum* strain CNCM I-2618 per daily dose. It may also comprise between 10^6 and 10^{11} cfu and/or between 10^6 and 10^{11} cells of *B. longum* strain CNCM I-2618 per g of the dry weight of the composition.

SUGARS

The *B. longum* strain CNCNCM I-2618 may be cultured in a medium comprising lactose, fructose or raffinose, or mixtures thereof at a concentration of, for example, 0.02 to 0.50 wt %. For example, the *B. longum* strain CNCM I-2618 may be cultured in a medium comprising lactose, fructose or raffinose, or mixtures thereof, at a concentration 0.05 to 0.15 wt %, 0.08 to 0.12 wt %, or about 0.10%.

Lactose is a disaccharide found in milk and composed of glucose and galactose. Fructose is a monosaccharide found in many plants. Raffinose is a trisaccharide also found in plants and composed of galactose, glucose and fructose.

The lactose, fructose, raffinose, or mixtures thereof, may be added to a conventional culture medium comprising up to 8% of another sugar suitable to sustain *B. longum* growth, such as, but not limited to, glucose or sucrose. Conventional culture medium suitable for growth of *B. longum* are well known to the person skilled in the art.

In one embodiment, the *B. longum* strain CNCM I-2618 may be cultured in a medium comprising the lactose, fructose or raffinose, or mixtures thereof, at a concentration of 0.03 to 0.40, 0.04 to 0.30 or 0.05 to 0.20 wt %.

In one embodiment, the culture medium may comprise the lactose, fructose or raffinose, or mixtures thereof, at a concentration of 0.03 to 0.15, 0.04 to 0.15, 0.05 to 0.15, 0.06 to 0.15 0.07 to 0.15, 0.08 to 0.15, 0.09 to 0.15 or 0.10 to 0.15 wt %.

In one embodiment, the culture medium may comprise the lactose, fructose or raffinose, or mixtures thereof, at a concentration of 0.05 to 0.14, 0.05 to 0.13, 0.05 to 0.12 or 0.05 to 0.11 wt %.

In one embodiment, the culture medium may comprise the lactose, fructose or raffinose, or mixtures thereof, at a concentration of 0.06 to 0.14, 0.07 to 0.13, 0.08 to 0.12, 0.09 to 0.11 or about 0.10 wt %.

In one embodiment, lactose is used at the concentrations described above.

In one embodiment, fructose is used at the concentrations described above.

In one embodiment, raffinose is used at the concentrations described above.

Process for producing a spray-dried culture powder

Strains belonging to the species *B. longum* are grown in anaerobic conditions. Fermentation methods under anaerobic conditions are commonly known. The skilled person is able to identify suitable components of the fermentation medium and to adjust fermentation conditions based on his general knowledge, depending on the microorganism to be grown. The fermentation medium typically comprises

- a nitrogen source such as yeast extract,
- a carbon source such as a sugar,
- various growth factors (e.g minerals, vitamins etc.) required by the microorganism and
- water.

A non-limiting example of a typical growth medium for *B. longum* is MRS (De Man, Rogosa and Sharpe) medium, supplemented with of cysteine (MRSc).

The fermentation is preferably carried out in two steps, a starter fermentation being carried out prior to the main fermentation step. The fermentation medium can be different for the starter and the main fermentation or may be identical.

The second step of the process is the concentration of the biomass. This can also be carried out using methods known to the person skilled in the art, such as for example centrifugation or filtration. The total solid content of the biomass after concentration is preferably comprised between 10 and 35wt%, preferably between 14 and 35wt%, based on the total dry weight of the biomass (i.e. of the total amount of fermentation medium and produced microorganism).

Optionally, the concentration may be preceded or combined with a washing step to remove residues of the fermentation medium and/or compounds produced during fermentation. For example, washing may be performed by concentrating biomass, re-suspending the concentrated biomass in a buffer, such as a phosphate buffer, or a similar composition and re-concentrating the biomass.

For example, the process described in WO2017/001590, which is entirely incorporated by reference, can be applied.

Spray drying

The conditioned biomass is then dried, by spray-drying. Spray drying is a conventional method of producing a dry powder by rapidly drying with a hot gas, well known in the art. Suitable heated drying mediums for spray-draying include air and nitrogen. The skilled person is able to identify suitable spray-drying conditions based on his knowledge. For example, the conditions described in EP0818529, which is entirely incorporated by reference, can be applied to spray-drying process.

Treatment

The spray dried *B. longum* strain CNCM I-2618 according to the present invention, or a composition comprising the same, may be used in the treatment or prevention of conditions related to gluten sensitivity or involving reduced activity of serine protease inhibitors.

For example, the spray dried powder or composition of the invention may be used in the treatment or prevention of inflammatory bowel disease, celiac disease, non-celiac gluten sensitivity, gluten ataxia, dermatitis herpetiformis and wheat allergy.

Preferably the condition is a gluten related disorder. Gluten-related disorders encompass diseases triggered by gluten. The terms “conditions related to gluten sensitivity” and “gluten-related disorders” are used interchangeably herein. Gluten-related disorders include celiac disease, non-celiac gluten sensitivity, gluten ataxia, dermatitis herpetiformis and wheat allergy.

Celiac disease

Celiac disease is one of the most common immune mediated disorders. It is a worldwide condition and is prevalent especially in the United States and Europe where around 1 % of subjects had positive antibody tests. Celiac disease is a complex disorder which arises from a complicated interaction among various immunologic, genetic, and environmental factors. It is triggered by the digestion of wheat gluten and other related cereal proteins such as rye and barley proteins. Symptoms linked with celiac disease are growth retardation, irritability and pubertal delay in children, and many gastrointestinal symptoms like discomfort, diarrhoea, occult stool, steatorrhea flatulence.

Clinical evidence shows class II human leukocyte antigens (HLA-DQII), which strongly relate with celiac disease pathology, are expressed in about 95 % of celiac disease patients. In the intestinal lumen, gluten proteins are partially digested, forming proteolytic-resistant 33-mer gluten peptides. After crossing the small intestinal barrier, they are deamidated by transglutaminase 2 (TG2) with negative charges (Sollid, 2000, Annual review of immunology, 18(1), 53-81), which then bind to the positively charged binding sites of HLA-DQ2.5/8 (Dieterich et al., 1997, Nature medicine, 3(7), 797-801). HLA-DQ2.5/8 displaying those specific gluten peptides signals to helper T cells and other immune cells causing further damage in the small intestine. Antibodies against gluten proteins and autoantibodies to connective tissue components (TG2) also associated with celiac disease progression (Alaedini & Green, 2005, Annals of internal medicine, 142(4), 289-298).

Non-celiac gluten sensitivity

Non-celiac gluten sensitivity (also designated as non-celiac wheat sensitivity) is an emerging condition. It is defined as a clinical entity induced by the ingestion of gluten leading to intestinal

and/or extra-intestinal symptoms which could be improved by removing the gluten-containing foodstuff from the diet (Lundin & Alaedini, 2012). The pathogenesis of non-celiac gluten sensitivity is not yet well understood. It has been shown that except for gliadin (main cytotoxic antigen of gluten), other proteins/peptides present in gluten and gluten-containing cereals (wheat, rye, barley, and their derivatives) may play a role in the development of symptoms. Non-celiac gluten sensitivity is the most common syndrome of gluten-related disorders with prevalence rates between 0.5–13 % in the general population (Catassi et al., 2013, *Nutrients, 5*(10), 3839-385). The diagnosis of non-celiac gluten sensitivity is made by exclusion of other gluten-related disorders.

Dermatitis herpetiformis

Dermatitis herpetiformis is a chronic blistering skin autoimmune condition, characterized by the presence of skin lesions that have an extensive and symmetrical distribution, predominating in areas of greater friction, and affecting mainly both elbows, knees, buttocks, ankles, and may also affect the scalp and other parts of the body. The lesions are vesicular-crusted and when flake off, they evolve to pigmented areas or a chromic an intense burning, itchy and blistering rash.

The age of onset is variable. It may start in childhood and adolescence but can also affect individuals of both sexes indistinctly at any age of their lives.

People with dermatitis herpetiformis have different degrees of intestinal involvement, ranging from milder mucosal lesions to the presence of villous atrophy.

Wheat allergy

Gastrointestinal symptoms of wheat allergy are similar to those of celiac disease and non-celiac gluten sensitivity, but there is a different interval between exposure to wheat and onset of symptoms. Wheat allergy has a fast onset (from minutes to hours) after the consumption of food containing wheat and can lead to anaphylaxis.

Gluten ataxia

Gluten ataxia is a gluten-related disorder. With gluten ataxia, damage takes place in the cerebellum, the balance center of the brain that controls coordination and complex movements

like walking, speaking and swallowing. Gluten ataxia is the single most common cause of sporadic idiopathic ataxia. It accounts for 40% of ataxias of unknown origin and 15% of all ataxias.

Gluten ataxia is an immune-mediated disease triggered by the ingestion of gluten in genetically susceptible individuals. It should be considered in the differential diagnosis of all patients with idiopathic sporadic ataxia. The effectiveness of the treatment depends on the elapsed time from the onset of the ataxia until diagnosis. The death of neurons in the cerebellum as a result of gluten exposure of the subject is irreversible. Early diagnosis and treatment with a gluten free diet can improve ataxia and prevent its progression. Less than 10% of people with gluten ataxia present any gastrointestinal symptom, yet about 40% have intestinal damage. Sensitive markers of gluten ataxia include anti-gliadin antibodies. Immunoglobulin A (IgA) deposits against transglutaminase 2 (TG2) in the small bowel and at extraintestinal sites are proving to be additionally reliable.

Administration

The spray dried Bifidobacterium longum strain CNCM I-2618 or composition of the invention are preferably administered enterally.

Enteral administration may be oral, gastric, and/or rectal.

In general terms, administration of the combination or composition described herein may, for example, be by an oral route or another route into the gastro-intestinal tract, for example the administration may be by tube feeding.

In an alternative embodiment administration of the combination or composition described herein may be topical administration.

The subject may be a mammal such as a human, canine, feline, equine, caprine, bovine, ovine, porcine, cervine and primates. Preferably the subject is a human.

Preferred features and embodiments of the invention will now be described by way of non-limiting examples.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, biochemistry, molecular biology, microbiology and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular

Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al. (1995 and periodic supplements) Current Protocols in Molecular Biology, Ch. 9, 13 and 16, John Wiley & Sons; Roe, B., Crabtree, J. and Kahn, A. (1996) DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; Polak, J.M. and McGee, J.O'D. (1990) In Situ Hybridization: Principles and Practice, Oxford University Press; Gait, M.J. (1984) Oligonucleotide Synthesis: A Practical Approach, IRL Press; and Lilley, D.M. and Dahlberg, J.E. (1992) Methods in Enzymology: DNA Structures Part A: Synthesis and Physical Analysis of DNA, Academic Press. Each of these general texts is herein incorporated by reference.

EXAMPLES

Example 1 – Serpin production

Production of powders

The main fermentation was performed in a 10 L Biostar B Plus fermenter containing 5.4% of dextrose and 0.1% of lactose. Live bacteria proportion and number were measured every hour by CytoFLEX to better adjust fermentation time. Bacteria were harvested after 14 h. Biomass was collected by centrifugation for 20 min at 5000 g. Protective agents (PA) (maltodextrin; sodium ascorbate; L-cysteine; L-alanine; L-lysine) were prepared in sterilized water and added in biomass with a ratio of 55 to 45 (dry mass ratio of PA/biomass) and stirred for homogenisation. Finally, half of it was subjected to spray drying and the other half to freeze drying. Total solid content (TS) was measured in biomass and biomass plus PA.

Spray drying was run in a Büchi system. The set parameters were presented as follow: Inlet temperature 165 °C and outlet temperature 80-83 °C. The system was preheated 20 min before.

Freeze-drying was performed in a Lyobeta 35 from Telstar. Freezing of the biomass was performed in liquid nitrogen. Primary drying was performed at 0.4 mbar, -15°C for 50 minutes. Secondary drying was performed at 20°C for 19h. Dried material was finally refined to obtain a fine powder.

Serpin mRNA detection and quantification

Serpin mRNA levels were measured in the frozen biomass (frozen *B. longum* CNCM I-2618, no drying process applied) and after drying.

Total RNA isolation – 500 µl of bacterial cultures were suspended in 1 ml of RNA protect bacteria reagent (Qiagen, Germany) and briefly centrifuged 10 min at 5000 g to harvest cells for total RNA extraction.

Total RNA was extracted using the Rneasy total RNA Mini kit (Qiagen, Germany, Ca.No. 74101) with protease K (Qiagen, Germany, Ca.No.19131) and further treated using Rnase-free Dnase set (Qiagen, Germany, Ca.No. 79254). Total RNA was then eluted in 40 µl water.

RNA quality control and quantification – Quality and quantity control of RNA samples were analysed by electrophoresis using QIAxcel Advanced System (Qiagen, Germany).

1 µl of RNA samples and RNA size marker were mixed separately with the same volume of RNA Denaturation Buffer and incubated 2 min at 70 °C, followed by 1 min cooling on ice. Then samples were diluted by QX RNA Dilution to 10 µl for analysing and measurement. Evaluation of RNA quality was performed using the ratio of 23S over 16S. RNA with a ratio value between 1.6 and 2.3 were selected for later qRT-PCR.

Both cDNA synthesis and PCR were performed in a one-step reaction, using the ABI Prism 7900HT system with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit with ROX (Invitrogen, cat. No. 11746-500).

PCR products were detected with SYBR green fluorescent dye and normalized with ROX reference dye.

The following primers were used for cDNA synthesis: for serpin gene: forward 5'-ACCAATCGCTGCTAAGTTCG-3', reverse 5'-TCGCTGGCAAGAGAGTAGTC-3'; for ldh: forward, 5'-CGAACGCCATCTACATGCTC-3' and reverse, 5'-AAGATCTGGTTCTCTTGCAG-3'. The primers for serpin were created based on *B. longum* NCC 2705 and DNA homology was checked in *B. longum* ATCC15707. The reliability was verified by dissociation curve analysis.

mRNA fold expression analysis method - The Pfaffl method was used to calculate relative transcription changes (Pfaffl, 2012) for which the equation is shown in Equation 1:

Equation 1

$$\text{Relative Quantity} = \frac{E_{ref\ target}^{CT_{ref\ target}}}{E_{ref\ calibrator}^{CT_{ref\ calibrator}}} \div \frac{E_{sample\ target}^{CT_{sample\ target}}}{E_{sampl\ calibrator}^{CT_{sample\ calibrator}}}$$

Final Colony Forming Units (CFU) after drying were equal to 1.79E11 and 1.49E11, respectively, for the freeze-dried and spray dried powder, representing a low viability loss upon drying of 0.2 log and 0.4 log, respectively. Spray dried powder presented much higher serpin mRNA level, as shown in Figure 1.

Example 2 – Inhibition of elastase

Primary sequence analysis showed that *B. longum* serpins are able to inhibit elastase-like serine proteases through the same mechanism than that of eukaryotic serpins (Ivanov et al., 2006; Journal of Biological Chemistry, 281(25), 17246-17252.; Roberts et al., 2004; Journal of molecular evolution, 59(4), 437-447). For example, Ivanov et al. explored the inhibitory activity of *B. longum* CNCM I-2618 (*B. longum* NCC 2705) serpin and found it could inhibit the activity of pancreatic elastase and neutrophil elastase.

Neutrophils activated by pathogenic bacteria at the site of intestinal inflammation release neutrophil elastase and other granule proteases which then exert antimicrobial activity (Burg & Pillinger, 2001; *Clinical immunology, 99*(1), 7-17). Production of protease inhibitors by bacteria may therefore play a role in protecting themselves against exogenous and innate proteases in the gastrointestinal tract. For example, *Bacillus brevis* survives external proteolytic attack through secretion of serine protease inhibitor BbrPI (Shiga et al., 1995, Bioscience, biotechnology, and biochemistry, *59*(12), 2348-2350).

It was therefore investigated whether spray dried *B. longum* CNCM I-2618 was associated with an increased ability to inhibit elastase.

Prototypes of *B. longum* CNCM I-2618 (*B. longum* NCC 2705) (frozen biomass, non-dried; freeze-dried (FD) and spray-dried (SD)) were tested in an animal model of gluten sensitivity (same model

than described in McCarville et al. 2017). Briefly NOD/DQ8 animals were sensitized and subsequently challenged with gluten. Probiotics were administered daily during the challenge at a dosis of 1E9 CFU per day. After the challenge, animals were sacrificed and intestinal content was collected for further elastase activity measurement.

As shown in Figure 2, the spray dried powder was surprisingly associated with greater elastase inhibition. Such activity may confer advantageous properties on the bacteria such as increased protection in the gastrointestinal tract.

PCT

Print Out (Original in Electronic Form)
(This sheet is not part of and does not count as a sheet of the international application)

0-1	**Form PCT/RO/134 Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared Using	CMS Online Filing Version CMS 1.15 MT/FOP 20020701/0.20.5.20
0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	15898-WO-PCT

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	17; 18; 3
1-2	**line**	5; 30; 31; 32; 1; 2; 6; 13
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
1-3-2	Address of depositary institution	Institut Pasteur 25-28, rue du Dr. Roux 75724 Paris Cédex 15, France
1-3-3	Date of deposit	29 January 2001 (29.01.2001)
1-3-4	Accession Number	CNCM I-2618
1-4	**Additional Indications**	
1-5	**Designated States for Which Indications are Made**	All designations
1-6	**Separate Furnishing of Indications** These indications will be submitted to the International Bureau later	

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0-4	**This form was received with the international application:** (yes or no)	Yes
0-4-1	Authorized officer	Kuiper-Cristina, Nathalie

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CLAIMS

1. Use of spray drying for increasing serpin expression in *B. longum* strain CNCM I-2618.

2. A process for increasing serpin concentration in a preparation of *B. longum* strain CNCM I-2618, wherein the process comprises spray drying the *B. longum* strain CNCM I-2618.

3. A process according to claim 1 wherein the process comprises growing *B. longum* strain CNCM I-2618 in a culture medium comprising a sugar selected from lactose, fructose and raffinose followed by spray drying the B. longum strain CNCM I-2618.

4. A process according to claim 1 or 2 wherein the a culture medium comprising the sugar selected from lactose, fructose and raffinose at a concentration of 0.02 to 0.5 wt %, 0.05 to 0.15 wt %, 0.08 to 0.12 wt %, or about 0.1%.

5. A composition comprising *B. longum* strain CNCM I-2618 in the form of a spray dried powder produced according to the process of any one of claims 2 to 4, for use in the treatment or prevention of inflammatory bowel disease, celiac disease, non-celiac gluten sensitivity, gluten ataxia, dermatitis herpetiformis or wheat allergy.

6. A composition for use according to claim 5 wherein the composition is a food, a medical food, a tube feed, a nutritional supplement, or a pharmaceutical composition wherein the pharmaceutical composition comprises one or more pharmaceutically acceptable carriers, diluents and/or excipients.

7. A composition according to claim 5 wherein the food is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, rice based products, milk based powders, infant formulae and pet food.

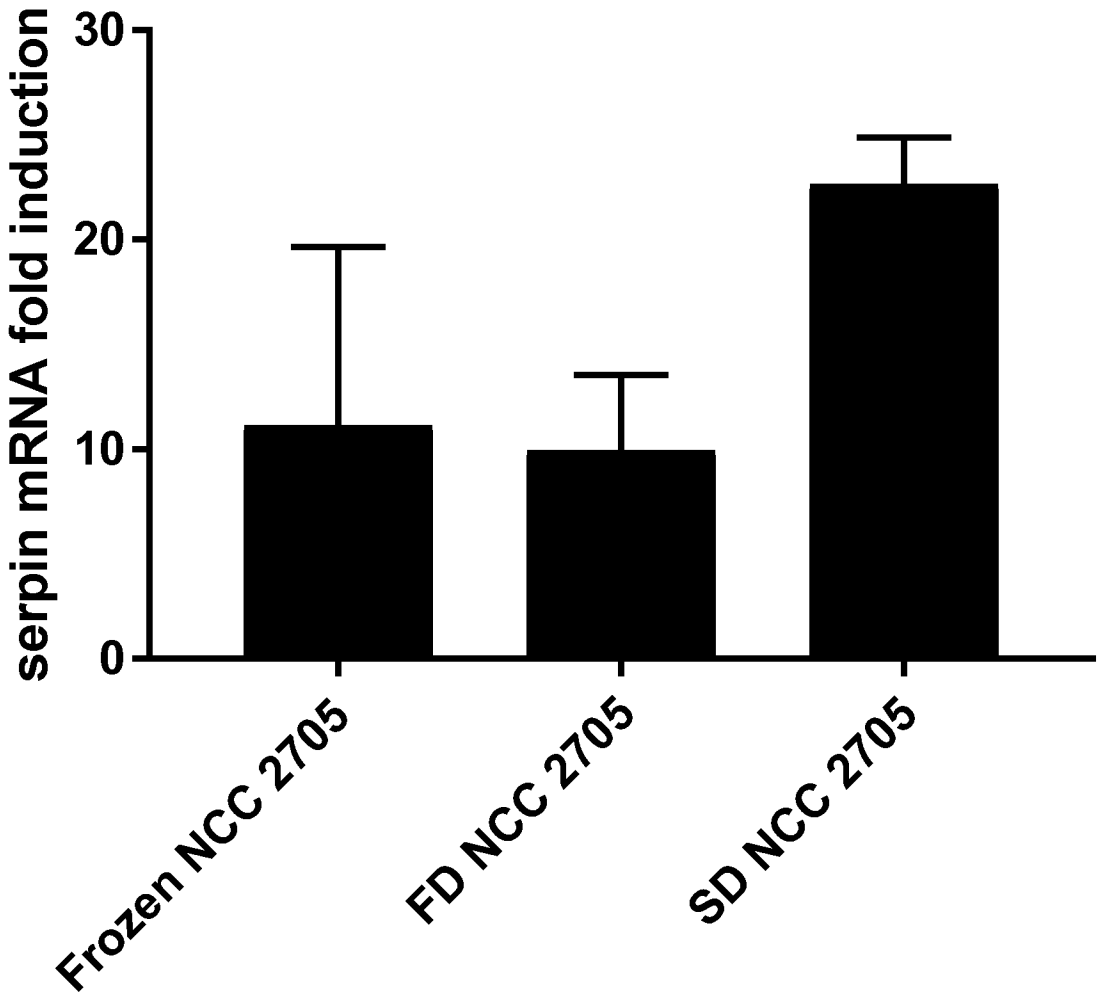

serpin mRNA fold induction
30
20
10
0
Frozen NCC 2705
FD NCC 2705
SD NCC 2705

Figure 1

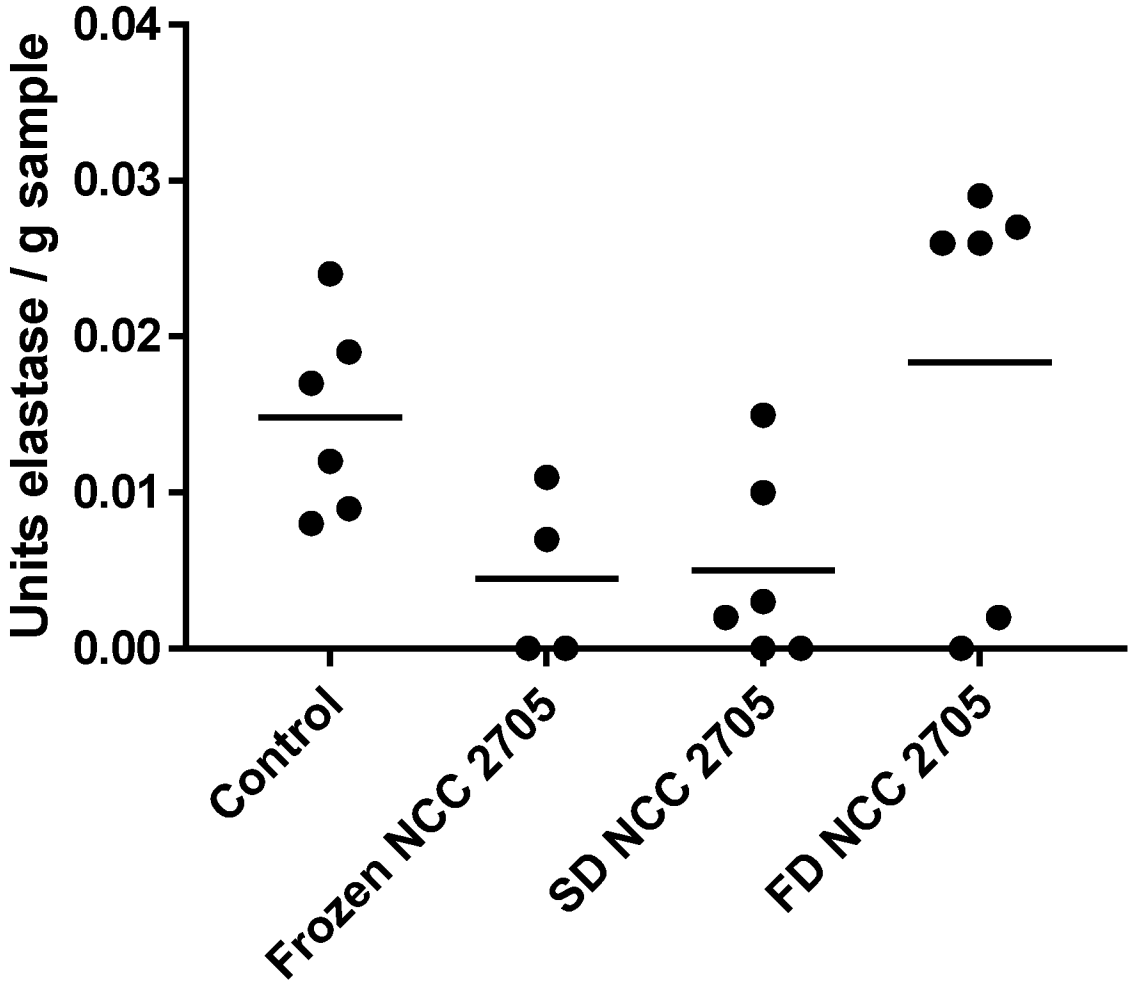

Units elastase / g sample
0.04
0.03
0.02
0.01
0.00
Control
Frozen NCC 2705
SD NCC 2705
FD NCC 2705

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/097021

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12R1/01 A61K35/74 C07K14/195 C12P21/02
C12N1/04 A23L33/135 A61K35/747
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)
C12N A61K C12R C07K C12P A23L

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	REIMANN S. ET AL: "Development of a rapid screening protocol for selection of strains resistant to spray drying and storage in dry powder", BENEFICIAL MICROBES, vol. 1, no. 2, 1 June 2010 (2010-06-01), pages 165-174, XP055474867, NL ISSN: 1876-2883, DOI: 10.3920/BM2009.0034	1-3
Y	the whole document	3-7
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 March 2019	25/03/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Kamp, Mart

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Form PCT/ISA/210 (second sheet) (April 2005)

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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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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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/097021

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