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WO-A1-2008/035332
WO-A1-2013/142792
WO-A1-2014/152484
WO-A1-2016/170285
WO-A2-2007/070052

Fortsættes ...

WO-A2-2012/016287

SEMYONOV D ET AL: "Microencapsulation of Lactobacillus paracasei by spray freeze drying", FOOD RESEARCH INTERNATIONAL, ELSEVIER APPLIED SCIENCE, BARKING, GB, vol. 43, no. 1, 1 janvier 2010 (2010-01-01), pages 193-202, XP026813715, ISSN: 0963-9969 [extrait le 2009-09-27]

TIAN HONGLIANG ET AL: "Freeze-dried, Capsulized Fecal Microbiota Transplantation for Relapsing Clostridium difficile Infection", JOURNAL OF CLINICAL GASTROENTEROLOGY UNITED STATES, USA, vol. 49, no. 6, 1 juillet 2015 (2015-07-01), pages 537-538, XP009189478, ISSN: 1539-2031, DOI: 10.1097/MCG.0000000000000330

BORODY THOMAS J ET AL: "Fecal Microbiota Transplantation: Expanding Horizons for Clostridium difficile Infections and Beyond", ANTIBIOTICS,, vol. 4, no. 3, 1 janvier 2015 (2015-01-01) , pages 254-266, XP009189481, YOUNGSTER ILAN ET AL: "Oral, capsulized, frozen fecal microbiota transplantation for relapsing Clostridium difficile infection", JAMA : THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, AMERICAN MEDICAL ASSOCIATION, UNITED STATES, vol. 312, no. 17, 5 novembre 2014 (2014-11-05), pages 1772-1778, XP009189475, ISSN: 1538-3598, DOI: 10.1001/JAMA.2014.13875

LIN ENMOORE ET AL: "Twelve Week Storage Trial of Microbial Viability in Lyophilized and Frozen Fecal Microbiota Preparations", GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, vol. 148, no. 4, Suppl.1, 1 mai 2014 (2014-05-01), page S962, XP009189480, ISSN: 0016-5085

LYOPHILISED COMPOSITION FOR PRESERVING MICROBIOTA IN ITS ECOSYSTEM

The present invention relates to the field of the preservation in the form of a lyophilisate of a microbiota in its ecosystem (MDSE). More particularly, it relates to a lyophilised composition of stools making it possible to preserve the faecal microbiota several months for the purpose of transplantation in an individual. The invention also relates to the therapeutic use in man or animals of such a composition, in particular in the form of capsules for an administration via the oral route.

Many studies show the interest of faecal microbiota transplantation (FMT) for therapeutically caring for certain pathologies that involve an imbalance in the faecal microbiota within its ecosystem, called dysbiosis, such as for example *Clostridium difficile* infections and their recurrences (Van Nood E. *et al.*, 2013). FMT already has a high clinical level of proof in such a way that recourse to this technique for this indication is mentioned in the most recent recommendations, whether European (Debast SB. *et al.*, 2014) or North American (Surawicz CM. *et al.*, 2013). The application of this technique to other pathological contexts of dysbiosis, such as inflammatory bowel diseases, irritable bowel syndrome, metabolic syndrome, obesity, diabetes, multi-resistant bacteria, even neuro-developmental disorders, of which the etiologies are uncertain or unknown, is also considered (Vrieze A *et al.*, 2013, Kelly CR. *et al.*, 2015).

Despite very strong growing interest for a few years now in the medical community and the recent emergence of patient requests, today there is no standardised procedure for carrying out FMT, whether for selecting the donor, the method of preparing the stools or the administration route.

Generally, the donor is selected based on a clinical examination and screening for a substantial panel of pathogenic agents such as bacteria, viruses or parasites, conducted upstream of the donation; this results in a validation rate of potential donors of less than 20% (Borody T. *et al.*, 2015). This, added to the delay for obtaining the results for the detection of pathogenic agents, greatly limits the carrying out of useable donations.

The particulars for administering a FMT are diverse. Faecal microbiota can be administered by nasogastric or nasojejunal tube, during a colonoscopy or by rectal enema (Kelly CR. *et al.*, 2015), with each one of these methods having its own advantages and disadvantages. The nasogastric or nasojejunal route, for example, can be badly perceived by the individual to be treated, with a risk of vomiting, even aspiration and can require a radiological monitoring when the tube is installed. The colonic route has the interest of

allowing for the observation of the mucosa at the same time as administration but it has factors of morbidity and a significant cost. Finally, the rectal enema route is the easier to implement and the least expensive but it can be difficult for the individual to be treated to ensure retention of the material administered. To these difficulties are added the reticence from patients linked to the organoleptic natures of the preparation.

The current recommendations of the Groupe Français de Transplantation Fécale in the framework of *Clostridium difficile* colitis (Sokol H. *et al.*, 2015) and of the ANSM for clinical tests (ANSM, 2015) concern an administration within 6h following the emission of the stools. In order to facilitate access to the donation, preserved faecal preparations frozen at -80°C and therefore immediately available are proposed. Their effectiveness is similar to that observed for transplantations using fresh material (Hamilton MJ. *et al.*, 2012; Youngster I. *et al.*, 2014a; Lee *et al.*, 2016). These frozen preparations require the carrying out of a dilution within a solution containing a cryoprotectant for protecting the microbiota from the effects of the freezing. The only cryoprotectant described in literature for the freezing of stools for the purpose of transplantation thereof is glycerol at the concentration of 10%. The effectiveness of the transplantation carried out with these frozen preparations containing glycerol is maintained up to 5 months post-preparation (Youngster I. *et al.*, 2014a).

The oral administration of capsules filled with this material and preserved at -80°C was reported with satisfactory results (Youngster I. *et al.*, 2014b; Youngster *et al.*, 2016). This type of preparation therefore opens new perspectives for an approach to dosing that facilitates the administration in the individual to be treated, in particular the patient. However, currently, it involves production that is still very empirical and requires preservation at -80°C to which the problems with storage are linked.

There is therefore an unsatisfied need to have stool preparations for which the preparing and the storing would be facilitated while still maintaining the initial qualities of the faecal microbiota during the preservation period and which would be available in a dosage form that allows for administration via a route that is first of all easy and non-invasive of a reduced volume.

In this context, the inventors have now developed a lyophilised composition comprising stools diluted in an optimised mixture of cryoprotectants with the purpose of facilitating the production of faecal preparations, their availability and their long-term

preservation, while still improving acceptability and comfort of the individuals to be treated.

Lyophilisation is a method of preservation used in the pharmaceutical industry for the preservation of bacterial strains. The lyophilisation has to be carried out in optimum conditions in order to ensure the maintaining of the viability as well as the balance of the different bacterial genera, in particular in the case of complex mixtures as is the case for MDSE in general and stools in particular. Using suitable cryoprotectants makes it possible to ensure the full or at least partial survival of the bacteria during a freezing.

There are three categories of cryoprotectant molecules with regards to living organisms, in particular bacteria:

- molecules with a high molecular weight which remain in the extracellular medium, such as proteins and polysaccharides, which facilitate the aqueous flow from the cells during the freezing phase, thus limiting the formation of intracellular ice crystals. By increasing the viscosity of the extracellular medium, these molecules also reduce crystal growth;

- molecules that are able to pass through the cell wall such as amino acids and oligosaccharides;

- molecules that penetrate the cell wall and the cytoplasmic membrane such as DMSO and glycerol (Carvalho A.S. *et al.*, 2004).

Penetrating agents of the second and third categories limit cellular dehydration and lower the freezing temperature inside the cell, thus reducing the saline concentration and reducing the osmotic shock (Béal C. *et al.*, 2015).

In order to optimise the preservation of the living organisms that form the faecal microbiota, the inventors have evaluated several cryoprotectants, individually or in combination. Glycerol at 10% conventionally used for FMTs carried out with frozen material (Hamilton MJ. *et al.*, 2012; Youngster I. *et al.*, 2014a) does not appear suitable for the production of lyophilisate. Among the cryoprotectants tested, two have shown to be promising: maltodextrin and trehalose. Although trehalose gives effective results, the results obtained with a mixture of maltodextrin and trehalose are even better for maintaining the viability of the microbiota (Figures 1 and 2) and for maintaining the diversity thereof (Figure 5). Maintaining the diversity is in particular very important due to the fact that the very purpose of the FMT is to re-establish the balance between the various populations present in the intestinal ecosystem of the benefitting individual. The

protective effects of the mixture of maltodextrin and trehalose with regards to the microbiota are observed for at least 3 months and up to 7 months after lyophilisation (Figures 3, 6 and 7). The mixture of maltodextrin and trehalose is also more favourable for maintaining the viability of extremely oxygen sensitive bacteria (EOS) such as the bacteria of the *Clostridium leptum* group (Figures 4 and 8). In addition, the concentration in bacterial metabolites such as short-chain fatty acids representative of the functional capacities of the faecal microbiota is preserved and even increased within the lyophilisate compared to the native stool (Figure 9). Finally, a direct anti-*Clostridium difficile* activity of the lyophilisates is revealed *in vitro* with inhibition of bacterial growth of 2 strains of *Clostridium difficile*, one toxinogenic (strain 630) and the other non-toxinogenic (strain PCD1) (Figure 10).

The object of the present invention is defined by claims 1-10.

Thus, the description relates to the use of a mixture of cryoprotectants for preserving a microbiota in its ecosystem (MDSE) in lyophilised form, characterised in that said mixture of cryoprotectants comprises maltodextrin (M) and trehalose (T) in an M/T weight ratio of between 35/65 and 45/55.

Maltodextrin is a polysaccharide cryoprotectant that does not penetrate the cell walls while trehalose is an oligosaccharide cryoprotectant that penetrates the cell walls.

The term "microbiota in its ecosystem" or MDSE, means a microbiota of which the composition is not modified with respect to that existing in the natural state in the organism. The microbiota and its ecosystem constitutes a complex and rich mixture, constituted in particular of bacteria, mushrooms, viruses, bacteriophages but also cellular debris, many peptides and proteins secreted by the enterocytes and cells of the *lamina propria* with their excretion/secretion products such as the secretory IgAs, calprotectin, cytokines and other anti-inflammatory or antimicrobial peptides of the defensin type, bacterial products secreted or not, including metabolites such as short-chain fatty acids, bacteriocins, and all the other biologically active molecules produced by the bacteria or other microorganisms of the microbiota, mucus, glucidic compounds of the mono and polysaccharide type, trace elements. The MDSE therefore constitutes a complex matrix that can be qualified as living, wherein many molecular and cellular interactions take place.

MDSEs can be preserved in lyophilised form by using a mixture of maltodextrin and trehalose according to the description. By way of examples, the MDSEs that can be

preserved according to this method are intestinal microbiota, formerly called intestinal flora, faecal microbiota contained in stools and vaginal microbiota formerly called vaginal flora. Each one of these microbiota is characterised by dominant bacterial groups well known to those skilled in the art.

5 Thus, the invention relates to the use of a mixture of cryoprotectants for preserving a microbiota in its ecosystem in lyophilised form, characterised in that said mixture of cryoprotectants comprises maltodextrin (M) and trehalose (T) in an M/T weight ratio comprised between 35/65 and 45/55, the microbiota in its ecosystem being intestinal microbiota, faecal microbiota or vaginal microbiota.

10 In an embodiment the MDSE to be preserved consists of stools.

In an embodiment, the invention relates to the use of a mixture of cryoprotectants such as described hereinabove, wherein the microbiota in its ecosystem consists of stools.

The term "lyophilisation" means, in terms of the description, the method that consists of removing water from a product using freezing then evaporation in a vacuum of the ice without passing through the liquid state, called sublimation. This technique is well known to those skilled in the art. There are three major phases in a lyophilisation cycle:

- freezing, where the products are refrigerated at temperatures of about -20 °C to -80 °C; the water is then transformed into ice;
- 20 - primary drying, in a vacuum, which consists of subliming the free interstitial ice;
- secondary drying, which makes it possible to extract via desorption the water molecules trapped on the surface of the dried products.

The term "stools" means, in terms of the description, the materials obtained after the digestion and absorption phases of foods and their transit in the intestinal tract. They are normally constituted of water at 75-85% by weight and dry matter at 15-25% by weight [desquamated intestinal cells and immune cells issues of *lamina propria* with their excretion/secretion products of the secretory IgAs type, calprotectin, cytokines and other anti-inflammatory or antimicrobial peptides, etc., elements that constitute microbiota (bacteria, virus, champignons, bacteriophages) and bacterial products secreted or not, including metabolites such as short-chain fatty acids, bacteriocins, and all the other biologically active molecules produced by the bacteria or other microorganisms of the microbiota: mucus; cellular debris of which the fibres and the cellulose that come from the non-digestible part of plants, lipids, iron, trace elements, etc.]. The stools represent,

in terms of the description, stools freshly produced by an individual in good health. Selecting donors in humans and preparing stools can be carried out according to the protocol and the conditions defined in the recommendations of the Groupe Français de Transplantation Fécale (Sokol *et al.*, 2015), in the article entitled "Practical
5 implementation of faecal transplantation" of Kapel N. *et al.*, 2014 and in the recommendations of the ANSM (2015).

In the mixture of cryoprotectants used to preserve in lyophilised form according to the description, maltodextrin (M) is used with trehalose (T) in an M/T weight ratio of between 35/65 and 45/55, and preferably of 40/60.

10 According to a preferred embodiment of the description, the cryoprotectants present in the mixture are exclusively maltodextrin and trehalose.

According to another embodiment of the description, the cryoprotectants present in the mixture include maltodextrin and trehalose as well as at least one other cryoprotectant chosen from mannitol, sorbitol, fructose, glucose, maltose, saccharose,
15 gelatine, starch, foetal calf serum and milk.

Another object of the description relates to a lyophilised composition comprising a microbiota in its ecosystem (MDSE) and a mixture of cryoprotectants, said mixture of cryoprotectants comprising maltodextrin (M) and trehalose (T), used in an M/T weight ratio of between 35/65 and 45/55, and preferably in a weight ratio of 40/60.

20 In an embodiment, the invention relates to a lyophilised composition comprising a microbiota in its ecosystem and a mixture of cryoprotectants, characterised in that said mixture of cryoprotectants comprises maltodextrin and trehalose, used in an M/T weight ratio of between 35/65 and 45/55, the microbiota in its ecosystem being intestinal microbiota, faecal microbiota or vaginal microbiota.

25 In the composition of the present description, maltodextrin is used with trehalose in an M/T weight ratio of between 35/65 and 45/55, and preferably in a weight ratio of 40/60.

According to preferred embodiment of the description, the cryoprotectants present in the lyophilised composition are exclusively maltodextrin and trehalose.

30 According to another embodiment of the description, the cryoprotectants present in the lyophilised composition include maltodextrin and trehalose as well as at least one other cryoprotectant chosen from mannitol, sorbitol, fructose, glucose, maltose, saccharose, gelatine, starch, foetal calf serum and milk.

The lyophilised composition according to the description contains a mixture of MDSE and cryoprotectants in a weight content from 15 to 35% and from 85 to 65% of the lyophilised composition respectively, preferably in a weight content from 18 to 28% and from 82 to 72%. These weight contents can vary according to various parameters, in particular the degree of hydration of the sample of MDSE as well as its content in lipids, in fibre and in cellular debris which can vary, in particular in the case of stools. Those skilled in the art will know how to adapt these weight contents in order to optimise the composition to be lyophilised.

Thus, the inventors have revealed that the lyophilisation of a composition comprising stools and a mixture of cryoprotectants, comprising maltodextrin and trehalose, makes it possible to preserve the viability and the biodiversity of the faecal microbiota over an extended period of at least 6 months. They also revealed a preservation of the production of bacterial metabolites in the lyophilisates. Furthermore, they have shown the capacity of lyophilisates in inhibiting the development of *Clostridium difficile* *in vitro*.

The term "viability of the faecal microbiota" means, in terms of the description, the part of the faecal microbiota that is alive.

The "biodiversity of the faecal microbiota" is represented by the various microbial populations present in the MDSE. The maintaining of constituent populations can be evaluated by the stability of representative groups of micro-organisms within the microbiota.

In a preferred embodiment, the present description has for object a lyophilised composition comprising stools and a mixture of cryoprotectants comprising maltodextrin (M) and trehalose (T) with an M/T weight ratio of between 35/65 and 45/55, preferably in a weight ratio of 40/60.

In this preferred embodiment wherein the MDSE consists of stools, the quantity by weight of stools represents 15 to 35% of the lyophilised composition and the quantity by weight of the mixture of cryoprotectants represents 85 to 65% of the lyophilised composition.

In yet a further preferred embodiment wherein the MDSE consists of stools, the quantity by weight of stools represents 15 to 35% of the lyophilised composition, the quantity by weight of the mixture of cryoprotectants represents 85 to 65% of the lyophilised composition and the mixture of cryoprotectants comprises maltodextrin (M)

and trehalose (T) in an M/T weight ratio of between 35/65 and 45/55, preferably in a weight ratio of 40/60.

In an embodiment, the invention relates to a lyophilised composition comprising a microbiota in its ecosystem and a mixture of cryoprotectants, characterised in that said mixture of cryoprotectants comprises maltodextrin and trehalose, used in an M/T weight ratio of between 35/65 45/55, wherein (i) the microbiota in its ecosystem (MDSE) consists of stools, (ii) the quantity by weight of stools represents 15 to 35% of the lyophilised composition and the quantity by weight of the mixture of cryoprotectants represents 85 to 65% of the lyophilised composition and (iii) maltodextrin (M) and trehalose (T) are used in an M/T weight ratio of 40/60.

In addition to ensuring the maintaining of the bacterial load, its viability and its diversity by preserving the balance between the dominant bacterial groups belonging to the phylum Firmicutes and Bacteroidetes, the composition according to the description has a serious advantage with respect to mixtures of bacterial strains (Allen-Vercoe E. *et al.*, 2013). Indeed, the composition of stools according to the description makes it possible to preserve the faecal microbiota in its nature original ecosystem (faecal ecosystem). The interactions between the various constituents as well as the functional capacities of this complex material are thus preserved and the individual who receives the FMT then benefits from the same advantages as during a transplantation of fresh stools or the administration of a bacterial filtrate (Ott *et al.*, 2016).

The term "faecal ecosystem" means, in terms of the description, everything that the stools are comprised of, i.e., not only the bacteria, viruses, mushrooms and bacteriophages but also the bacterial products secreted or not, including metabolites such as short-chain fatty acids, bacteriocins, and all the other biologically active molecules produced by bacteria or other microorganisms of the microbiota as well as desquamated intestinal cells or in apoptosis (enterocytes, immune cells of the *lamina propria*) and the excretion/secretion products thereof (secretory IgAs, calprotectin, cytokines and other anti-inflammatory or antimicrobial peptides, etc.), glucidic elements of the mono- and polysaccharide type such as fibres and cellulose that come from the non-digestible part of plants, protein and lipid residues, trace elements, mucus, etc.

The composition of the description is particularly suited for oral administration.

It can, for example, have the form of a gastro-resistant capsule or tablet, of granules that do not require rehydration or a spray, according to the individual to be treated.

According to a particular embodiment of the description, the lyophilised composition has the form of a gastro-resistant capsule or tablet. This presentation is particularly suited for oral administration in humans. The term "gastro-resistant tablet or capsule" means, in terms of the description, an oral dosage form that resists gastric juices and that is enterically coated.

These formulations can furthermore contain a pharmaceutically acceptable vehicle in order to render lyophilised composition suitable for administration in the subject to be treated. This vehicle is preferably inert, and in any case, of a nature to maintain the viability and the diversity of the microbiota within its ecosystem and to release the lyophilised composition at the suitable site.

Thus, the present invention also has for object a gastro-resistant capsule comprising the lyophilised composition such as defined hereinabove.

The filling of said capsules, whether manually or industrially, is carried out by volume. For this it is necessary that the lyophilisate be sprayed and that the powder to be distributed has good fluidity in order to ensure regular filling of the capsules (Le Hir et al., 2016).

The flow properties of a powder can be evaluated according to the "2.9.16. Flow" test of European pharmacopoeia (European Pharmacopoeia., 2016) which measures the time required for the flow of a test sample through the orifice of a funnel of standardised dimensions. A time less than or equal to 10 seconds/100 g of powder corresponds to an excellent flow. On the contrary, the flow time for a powder that does not flow will be noted as "infinite". This is the case of a raw faecal lyophilisate which therefore cannot be used as is for the filling of capsules.

In order to overcome this limit, the inventors have developed capsules comprising the lyophilised composition such as defined hereinabove with the addition of flow excipients such as diluents and flow lubricants, able to improve the fluidity of faecal lyophilisates.

In an embodiment, the invention relates to a gastro-resistant capsule such as described hereinabove, characterised in that said lyophilised composition is mixed with at least one flow excipient.

These flow excipients used in filling capsules with lyophilisates of the description are chosen from mineral excipients, such as dicalcium phosphate, maltodextrins, talc, a hydrophilic colloidal silica and a hydrophobic colloidal silica.

Thus, according to the description, the gastro-resistant capsule comprises a lyophilised composition such as defined hereinabove, mixed with at least one flow excipient chosen from a flow lubricant and/or a diluent for direct compression.

5 According to a particular embodiment, the flow excipient is a flow lubricant used in a quantity that is compliant with conventional use; preferably, the quantity of lubricant mixed with the lyophilisate is between 0.1 and 1% by weight of the lyophilisate and lubricant mixture, more preferably this quantity of lubricant is 0.5% by weight of the lyophilisate and lubricant mixture.

10 The flow lubricant can be chosen from talc, hydrophobic colloidal silica, hydrophilic colloidal silica; preferably, it is hydrophobic colloidal silica.

According to another particular embodiment, the flow excipient is a diluent for direct compression used in a quantity that is compliant with conventional use, preferably, the quantity of diluent mixed with the lyophilisate is between 10 and 90% by weight of the lyophilisate and diluent mixture, more preferably between 15 and 75% or 25 and 50%
15 by weight of the lyophilisate and diluent mixture.

The diluent for direct compression can be chosen from dicalcium phosphate or nebulised maltodextrins,

20 According to yet another particular embodiment, the flow excipient comprises a flow lubricant and a diluent for direct compression such as defined hereinabove and in the proportions indicated.

By way of example, the lyophilisate of MDSE and flow excipient mixture is comprised of:

25 - a hydrophobic colloidal silica between 0.1 and preferably 0.5%, by weight in relation to the total weight of the mixture;

- a hydrophilic colloidal silica between 0.1 and 1%, preferably 0.5%, by weight in relation to the total weight of the mixture and dicalcium phosphate between 10 and 90%, preferably between 15 and 75%, preferably 25 to 50%, by weight in relation to the total weight of the mixture;

30 - talc between 0.1 and 1%, preferably 0.5%, by weight in relation to the total weight of the mixture and nebulised maltodextrins between 10 and 90%, preferably between 15 and 75%, by weight in relation to the total weight of the mixture.

In this context, the development of gastro-resistant capsules prepared from a lyophilised composition constitutes definite progress in terms of organoleptic

characteristics of the preparation, availability of the material to be transplanted, preservation of the preparation and comfort of the individual to be treated.

Lyophilisation makes it possible to remove the water present in the stools. Indeed, normal stools contain between 75 and 85% water. After lyophilisation, 50g of stools corresponds to about 7.5-12.5g of lyophilised microbiota to which the cryoprotectants are added.

In comparison with the administration of stools according to conventional methods, whether entailing fresh stools or frozen stools, administration in the form of a lyophilisate that can be included in gastro-resistant capsules therefore facilitates the standardisation of the preparation and its acceptability by the individual to be treated. Thus, morbidity should be decreased and the cost of producing the faecal transplantation will be reduced when oral administration in the form of a lyophilisate is well-established in medical practice. Indeed the oral administration of FMT in the form of a lyophilised preparation will no longer be associated with technical gestures that require specialised personnel and which require day hospitalisation, as is currently the case for administration via the naso-duodenal, colonoscopic or rectal route of preparations coming from fresh or frozen stools. Compared to the administration of frozen capsules, administration in the form of a lyophilisate will facilitate the manufacturing and storage process, and therefore the standardisation of the preparation. Furthermore, using lyophilised preparations will allow for improved accessibility of the material required for FMT, a crucial point in order to respond to emergency situations that can be encountered, for example, in the framework of *Clostridium difficile* infection recurrences.

Thus, the present description also has for object a method for preserving stools by lyophilisation comprising the use of a mixture of cryoprotectants such as defined hereinabove. This method makes it possible to preserve the lyophilised stools at least for 3 months and for a period that range up to 7 months, even longer by ensuring the maintaining of the viability and of the diversity of the microbiota as described above.

The lyophilised preserved stools according to the description can be administered in the framework of an allogeneic or autologous FMT. Indeed, the FMT can consist of a transfer of stools between two individuals of the same species or of an administration to the same initially healthy individual donor at a given time when he becomes an individual to be treated at a later time. Thus, an individual in good health can later have the

lyophilised composition comprising a preparation of stools coming from his donation, at the time when he needs it.

The present description has for other object a lyophilised composition or a gastro-resistant capsule such as defined hereinabove for its use as a drug.

5 The present description thus relates to the lyophilised composition or to the gastro-resistant capsule described hereinabove for its use in the treatment of a disease or a disorder linked to an imbalance of intestinal microbiota.

10 Indeed, intestinal microbiota constitutes an ecosystem in balance and which is constantly regulating itself. This balance is threatened by aggressions that can result in the rupture thereof. Dysbiosis is defined as a loss in the diversity or an imbalance in the microbiota which can result in an excess of deleterious microorganisms and/or in a relative insufficiency of microorganisms that are beneficial to the host. Thus, the indications, for the use of the lyophilised composition according to the description are those which are associated with a dysbiosis. As such, as "imbalance-related disease or
15 order of the intestinal microbiota", mention can be made of *Clostridium difficile* colitis and its recurrences, chronic inflammatory diseases of the intestine such as Crohn's disease and haemorrhagic rectocolitis, irritable bowel syndrome, the carrying of multi-resistant bacteria, constipation, traveller's diarrhoea, digestive infections, autoimmune diseases, diverticulosis, type 1 and type 2 diabetes, metabolic syndrome, obesity, rheumatoid
20 polyarthritis, ankylosing spondylitis and sacroiliitis, chronic fatigue syndrome, halitosis, allergies, acne, insomnia, depression and neuropsychiatric diseases such as for example, Parkinson's disease and illnesses on the autism spectrum.

 In an embodiment, the invention relates to a lyophilised composition or to a gastro-resistant capsule described hereinabove for its use in the treatment of a disease or a
25 disorder linked to an imbalance of intestinal microbiota chosen from *Clostridium difficile* colitis and its recurrences, chronic inflammatory diseases of the intestine such as Crohn's disease and haemorrhagic rectocolitis, irritable bowel syndrome, the carrying of multi-resistant bacteria, constipation, traveller's diarrhoea, digestive infections, autoimmune diseases, diverticulosis, type 1 and type 2 diabetes, metabolic syndrome, obesity,
30 rheumatoid polyarthritis, ankylosing spondylitis and sacroiliitis, chronic fatigue syndrome, halitosis, allergies, acne, insomnia, depression and neuropsychiatric diseases such as Parkinson's disease and illnesses on the autism spectrum.

Preferably, the lyophilised composition of the description is used in the treatment of *Clostridium difficile* colitis. In this context, the presence of bacterial metabolites preserved in the lyophilisates can have a major beneficial effect for fighting *C. difficile*.

5 The lyophilised composition can also be used in the treatment of chronic intestinal inflammatory diseases, the carrying of multi-resistant bacteria, type 1 and type 2 diabetes, metabolic syndrome, obesity and illnesses on the autism spectrum.

The preservation of the environment associated with the microbiota can have a major contributory effect in the therapeutic effect of FMT.

10 The lyophilised composition or gastro-resistant capsule according to the description can furthermore be used as a back-up to oral antibiotic treatment, such as metronidazole or vancomycin (first-line treatment in *Clostridium difficile* infections) in particular for the treatment of *Clostridium difficile* colitis recurrences, where the individuals have become insensitive to a repeated treatment via antibiotics.

15 In an embodiment, the invention relates to a lyophilised composition or the gastro-resistant capsule for its use such as described hereinabove, as a back-up to oral antibiotic treatment, in case of recurrences of *Clostridium difficile* infections.

A composition of lyophilised MDSE according to the description is intended to be administered to a human or an animal.

20 In a context where action plans on antibiotherapy aiming to limit the risk of the development of multi-resistant bacteria are being developed, using such a lyophilised composition constitutes an alternative choice in veterinary medicine, in particular in organic farms. Thus, the lyophilised composition according to the description can be used in humans, but also in animals such as pigs, cattle, sheep, rodent, lagomorphs, carnivores, equidae, birds, reptiles more particularly in pigs, cattle, chicken, rats, rabbits, dogs, cats, 25 horses. As FMT is a method that has been used empirically for a long time and is being developed in veterinary medicine, the administration of stools in lyophilised form is obviously of a nature to facilitate the implementation thereof.

30 In order to facilitate administration in animals, a lyophilised composition of MDSE can be prepared in the form of granules that do not require rehydration or spray. Those skilled in the art will know how to adapt the dosage form in such a way as to render the most suitable possible according to the animal to be treated.

Another object of the present description relates to a method for preparing a composition of lyophilised MDSE according to the description, comprising the following steps:

- collection of the MDSE, in particular of stools;
- 5 - dilution of the MDSE with a mixture of cryoprotectants such as defined hereinabove in an M/T weight ratio of between 35/65 and 45/55, and preferably in an M/T weight ratio of 40/60;
- lyophilisation of the mixture obtained in the preceding step and obtaining of the lyophilised composition.

10 According to a particular embodiment, lyophilisation can be carried out by applying a freezing temperature ranging from -20°C to -80°C, for a duration ranging from several tens of minutes to several hours. In a preferred embodiment, the step of freezing is carried out at -80°C for 30 min. Those skilled in the art will know how to adapt these conditions.

15 As indicated hereinabove, the mixture of MDSE and cryoprotectants is such that it comprises 15 to 35% by weight of MDSE and 85 to 65% by weight of cryoprotectants in relation to the total weight of the lyophilised composition, preferably a weight content of 18 to 28% and of 82 to 72%. These contents can vary according to various parameters, in particular the degree of hydration of the sample of MDSE as well as its content in lipids,
20 in fibre and in cellular debris which can vary, in particular in the case of stools. Those skilled in the art will know how to adapt these weight contents in order to optimise the composition to be lyophilised.

In a particular embodiment, the present description relates to a method for preparing a gastro-resistant capsule comprising a lyophilised composition according to
25 the description, comprising the following steps:

- preparing a lyophilised composition;
- spraying the lyophilisate;
- possibly adding one or more excipients facilitating the flow of the lyophilisate;
- filling of the case of the gastro-resistant capsule with said lyophilised mixture;
- 30 - closing of the capsules.

In an embodiment, the invention relates to a method for preparing a gastro-resistant capsule such as described hereinabove, comprising the following steps:

- a. collecting a microbiota in its ecosystem;

b. dilution of said microbiota in its ecosystem with a mixture of cryoprotectants comprising maltodextrin and trehalose in an M/T weight ratio of between 35/65 and 45/65;

c. lyophilisation of the mixture obtained in step b;

5 d. spraying of the lyophilisate obtained in step c;

e. possibly adding one or more excipients facilitating the flow of the lyophilisate;

f. filling of the case of the gastro-resistant capsule with said lyophilised mixture;

g. closing of the capsules,

10 the microbiota in its ecosystem being intestinal microbiota, faecal microbiota or vaginal microbiota.

In an embodiment, the invention relates to a method such as described hereinabove wherein said microbiota in its ecosystem consists of stools, and the mixture of cryoprotectants consists of a mixture of maltodextrin (M) and trehalose (T) in an M/T weight ratio of 40/60.

15 Particular embodiments are described in the following examples. Their only objective is to show the description and to allow it to be understood and must not in any case be considered as a limitation.

DESCRIPTION OF THE FIGURES

20 Figure 1: Representation of the main bacterial and yeast groups found after culture in the native stools and after dilution in NaCl 9‰ or in the mixture of cryoprotectants of maltodextrin at 6.7% and trehalose at 10% (MT). The results shown in figure 1 correspond to those obtained for the stool 3.

25 Figure 2: Representation with respect to the native stool of the viable bacterial load after reconstitution of the lyophilisate according to the nature of the cryoprotectant: maltodextrin 5% (M), trehalose at 5% (T5), trehalose at 10% (T10), mixture of maltodextrin at 6.7% and trehalose at 10% (MT) or milk (L).

30 Figure 3: Representation of the change in the bacterial load over time according to the nature of the cryoprotectants: trehalose at 5% (T5), trehalose at 10% (T10) and mixture of maltodextrin 6.7% and trehalose at 10% (MT). J0 represents the pre-lyophilisation; M0, the post-lyophilisation; M4, 4 months post-lyophilisation and M7, 7 months post-lyophilisation.

Figure 4: Representation of the percentage of EOS at M0 according to the nature of the cryoprotectant: maltodextrin at 5% (M), trehalose at 5% (T5), trehalose at 10% (T10), maltodextrin mixture at 6.7% and trehalose at 10% (MT). J0 represents the pre-lyophilisation; M0, the post-lyophilisation.

5 Figure 5: Representation of the main bacterial groups found in the native stool and in the lyophilisates immediately after preparation (M0) according to the nature of the cryoprotectant used: trehalose at 5% (T5), trehalose at 10% (T10) and mixture of maltodextrin at 6.7% and trehalose at 10% (MT). The results shown in figure 5 correspond to those obtained for the stool 3.

10 Figure 6: Representation of the main bacterial groups found in the native stool and in the lyophilisates after preparation according to the nature of the cryoprotectant used: trehalose at 5% (T5), trehalose at 10% (T10) and mixture of maltodextrin at 6.7% and trehalose at 10% (MT). The results are presented immediately after lyophilisation and after storage at 4°C for 3 months (+3) and 8 months (+8). The results shown in figure 6
15 correspond to those obtained for the stool 3.

 Figure 7: Representation of the impact of the lyophilisation (L72h) then of the preservation of the lyophilisates for 3 months (L3M) and 6 months (L6M) on the viability of the main bacterial groups of three faecal samples (S4, S5, S6) with respect to the native stool (SN). (A) Effect on different strict anaerobic bacterial populations. (B) Effect on
20 different optional aero-anaerobic bacterial populations.

 Figure 8: Representation of the impact of the lyophilisation (L72h) then of the preservation of the lyophilisates for 3 months (L3M) and 6 months (L6M) on the viability of extremely oxygen sensitive bacteria (EOS) of the various faecal samples (S4, S5, S6) with respect to the native stool (SN).

25 Figure 9: Evaluation of the production of bacterial metabolites by gas chromatography of native stools (SN) and lyophilised (L72h).

 Figure 10: Evaluation of the anti-*Clostridium difficile* effect of the native stools (S or SN) and lyophilised (L72h)

EXAMPLE 1

1. Equipment and Methods

1.1. Faecal samples

The selection of the lyophilisation conditions was carried out using the study of 3 stools coming from independent healthy volunteers and placed immediately after emission in the presence of a GENbagAnaer® system placed in the sample jar for incubation. Transmission to the laboratory was carried out immediately after emission, within a period of less than 1 hour. A fraction of each stool is preserved in GENbagAnaer® as a control for immediate analysis.

1.2. Choice of cryoprotectants

The cryoprotectants used for this study are as follows:

- glycerol at 10% (G).
- reconstituted powdered pasteurised skimmed milk at 10% (L),
- altodextrin, at the concentration of 5% (M).
- trehalose, at concentrations of 5% (T5) and 10% (T10).
- the mixture constituted of maltodextrin at a concentration of 6.7% and trehalose at a concentration of 10%, i.e. in an M/T weight ratio of 40/60 (MT).
- a solution of NaCl at a concentration of 9‰, used as a control as it is usually used to dilute the fresh faecal material.

Each solution was conditioned in sterile jars and preserved at +4°C until used.

1.3. Preparation of the faecal lyophilisate

The stools were diluted 10 times in the cryoprotectant solution and homogenised using a homogeniser of the Ultraturax® type equipped with a single-use rod. The suspensions were deposited in lyophilisation vials in a volume of 3 ml corresponding to 300 mg of native stool.

For each condition tested, and for each one of the 3 stools analysed, a suspension vial was immediately placed in the presence of a GENbagAnaer® system in order to evaluate the effect of the various cryoprotectants on the viability of the bacteria of the microbiota of each diluted and non-lyophilised stool.

The remaining vials are immediately placed at -80°C. After 30 minutes of freezing, the vials are placed in the lyophiliser (FreeZone® 6 Litre, Freeze Dry System, Model 77530,

Labconco Corporation, Kansas City, MO, USA) of which the trap is already cooled for a duration of 24h under an automatic cycle. At the end of this period of lyophilisation, the vials are plugged in a vacuum then crimped using aluminium capsules.

For each stool and each cryopreservation condition, a series of vials is transmitted
5 to the microbiology laboratory for analysis of the microbiota and 2 series are preserved at 4°C for stability studies at 3-4 months and 7-8 months.

1.4. *in vitro* studies of the viability of the microbiota according to the conditions of lyophilisation

10 The first step of the microbiological study concerned the analysis of the diluted non-lyophilised stools in order to test the effect of the dilution in the presence of the various cryoprotectants on the maintaining of the diversity of the microbiota and the viability thereof. For this, an aliquot of the dilutions carried out in a milk medium at 10% (L), glycerol 10% (G), maltodextrin 5% (M), trehalose 5% (T), trehalose 10% (T10) and mixture
15 of maltodextrin 6.7% and trehalose 10%, which is an M/T weight ratio of 40/60 (MT) was placed immediately after the preparation of faecal lyophilisate under anaerobic conditions and the microbial cultures were carried out immediately.

The second step of the work concerned the study of lyophilisates. The analyses were conducted within the week following the preparation then after 3-4 months and 7-
20 8 months of storage at 4°C. Each lyophilisate is reconstituted with 3 ml of sterile peptone water, which corresponds to the volume of diluted stool initially present in each vial.

For the analysis of strict anaerobic and aerobic microbiota, the non-lyophilised stools and the reconstituted lyophilisates were diluted from 10 to 10 and inoculated over different selective and non-selective mediums (Rougé *et al.*, 2010). The mediums
25 incubated in aerobic conditions and in anaerobic chamber (N₂/CO₂/H₂:80/10/10) at 37°C are indicated in table 1.

Table 1: Mediums used for the analysis of the microbiota per culture

Elements of the microbiota detected	Agar base	Incubation/reading
Total anaerobic counts	Colombia + cysteine (Cc) + blood + neomycin (100 mg/ml)	37°C Anaerobic chamber Reading and enumeration at 48h and 5 days
<i>Bacteroides</i> sp., <i>Prevotella</i> sp.	Cc + blood + kanamycin (100 mg/ml) + vancomycin (7 mg/ml)	
<i>Clostridium difficile</i>	Cc + blood + <i>Clostridium difficile</i> mixture (CLO-M Biomérieux)	
Other <i>Clostridium</i>	CC + mupirocin (100 mg/ml) + whole milk (5ml) + neutral red (40 mg/ml)	
<i>Bifidobacterium</i> sp.	WCB (Wilkins-Chalgren for Bifidobacteria) + mupirocin (100 mg/ml)	
<i>Lactobacillus</i> sp.	MRS (de Man, Rosaga, Sharpe agar)	
Total aerobic counts	Trypticase soy	37°C Aerobically Reading at 24h
Enterobacteria	Drigalski	
<i>Enterococcus</i> sp.	Enterococcosel	
<i>Staphylococcus</i> sp.	Chapman 110	
Yeasts	Sabouraud Chloramphenicol	

Quantification of the extremely oxygen sensitive bacteria (EOS) such as the bacteria in the *Clostridium leptum* group, was carried out by differential culture in the presence or absence of oxygen. For this, the samples of the fresh stool (T0) and the various lyophilised samples rehydrated in peptone water were successively diluted in a very rich non-selective culture medium (YBHI corresponding to a brain-heart medium enriched with yeast extract) supplemented with cellobiose, maltose and cysteine. This medium was developed in order to allow for the culture of bacteria that are extremely difficult to cultivate of the *Faecalibacterium prausnitzii* type. Once *ad hoc* dilutions are carried out in strict anaerobic conditions, their capacity to resist or not the presence of oxygen was evaluated by culture in 2 dishes in parallel, one being subjected to an aerobic atmosphere for 60 minutes and the other not. This method makes it possible to evaluate the percentage of strict aerobic viable bacteria within different samples, at different times after lyophilisation.

2. Results

2.1. Effect of the various cryoprotectants on the diversity and the viability of the microbiota before lyophilisation.

A dilution of the stool in the various cryoprotectants evaluated, and in particular the mixture of interest MT, did not have any significant impact on the bacterial load, or on the balance of the main genera that can be cultivated of the microbiota, compared to the native stool or the stool diluted in NaCl at 9‰ (corresponding to the preparation used for FMT with fresh stools) (Figure 1). The majority groups of the microbiota remain viable.

2.2. Effect of the lyophilisation on the viability of the microbiota according to the cryoprotectants used

The total bacterial load was evaluated in the week following lyophilisation, i.e. at M0. The results are shown in Figure 2. The three cryoprotectants tested, namely a trehalose solution at 5% (T5), a trehalose solution at 10% (T10) and a mixture of maltodextrin 6.7% and trehalose 10% (T10), make it possible to ensure a stability greater than 90% of the total bacterial load of the species that can be cultivated during lyophilisation; this results corresponds to a loss of about 1 log with respect to the existing load in a native stool or a stool diluted in NaCl at 9‰ before lyophilisation.

This total bacterial load, measured up to 7 months post-lyophilisation changed little (Figure 3).

In addition, these 3 cryoprotectants allows for an effective preservation of the EOS bacteria during lyophilisation (Figure 4).

2.3. Effect of the lyophilisation on the diversity of the microbiota according to the different cryoprotectants used

Among the 3 cryoprotectants tested hereinabove, the cryoprotectant mixture of maltodextrin at 6.7% and trehalose at 10%, (which is in a weight ratio of 40/60) is the one that gives the best results with regards to maintaining alive the main genera that constitute the faecal microbiota, despite a decrease in certain strict anaerobic genera of about 1 to 2 log, if stool 3 is considered (Figure 5) (and up to 4 log for the other stools - results not shown). Conversely, glycerol at 10% has shown to provide little protection, whether on aerobic genera or anaerobic genera (result not shown).

The strict anaerobic genera (*Clostridium* sp. and *Bacteroides* sp.) remain dominant when the study is carried out in the week following lyophilisation (Figure 6). Preserving the lyophilisate at 4°C has only little repercussion with an additional loss of about 1.5 log for *Bacteroides* sp. after 3 months of preservation at 4°C and a loss of 1 log at 8 months for *Clostridium* sp (Figure 6). In any case, the dominant genera of the microbiota remain in a state of dominance during preservation.

The results obtained with the stool 2, rich in *Bifidobacterium* sp. have shown the stability of this group, up to 6 months post-lyophilisation in the mixture MT (result not shown).

Taken as a whole, these results demonstrate that the mixture MT is the one that allows for the best preservation of the lyophilised faecal microbiota, in terms of viability as well as diversity and this for a duration that can range up to 6 months after lyophilisation.

EXAMPLE 2

1. Equipment and Methods

In application of the method described in example 1, three additional faecal samples identified as S4, S5 and S6 were diluted in the presence of the cryoprotectant mixture of maltodextrin at 6.7% and trehalose at 10% (i.e. in an M/T weight ratio of 40/60), then lyophilised.

2. Results

2.1. Stability of the bacterial ecosystem in the lyophilisates after preservation for 6 months

2.1.1. Analysis of the bacterial viability and diversity in lyophilisates after preservation for 6 months

The strict anaerobic population was analysed based on the enumeration of representative bacterial groups: *Bifidobacterium* spp. and *Bacteroides* spp.

Regarding the *Bifidobacterium* spp. genus, it remains an integral part of the dominant microbiota and the absence of a significant change over the period of preservation of 6 months is confirmed.

For the *Bacteroides* spp. genus, there are inter-individual differences that result in a loss of about 1.5 to 4 log at 6 months.

The optional aero-anaerobic populations of the various samples were analysed based on the enumeration of the bacterial groups *Lactobacillus* spp., *Enterococcus* spp. and *Enterobacteriaceae*. The stability and the viability of these bacterial genera and groups post-lyophilisation all throughout the period of preservation over 6 months are confirmed.

These results show that the lyophilisation process makes it possible to preserve for the most the viability of the different target bacterial populations without induction of modifications in the major balances between the main bacterial groups and that this preservation remains stable over a period of preservation of 6 months (Figure 7).

2.1.2. Study of extremely oxygen sensitive bacteria

One of the specificities of faecal microbiota is the presence of extremely oxygen sensitive bacteria (EOS) that can be affected by the lyophilisation process.

These EOS bacteria are detected both for the native stool and in the faecal lyophilisates for all the samples analysed (Figure 8). When these bacteria EOS are expressed as a percentage with respect to the total quantity of anaerobic bacteria (CFU/ml), their level remains stable all throughout the preservation (3 to 6 months according to the samples) with a quantity of strict anaerobic bacteria which itself is not affected by the preservation process.

These results show that the lyophilisation does not affect the bacterial populations that are highly sensitive to oxygen.

2.2. Stability of the functions of bacterial ecosystem in the lyophilisates

2.2.1. Detection of metabolites in the lyophilisates

The production of bacterial metabolites, namely of short-chain fatty acids, was evaluated in the lyophilisate of 72h in comparison with the native stool. Over the 3 samples analysed, the lyophilisation induces a preservation of the fermentation products with a concentration in short-chain fatty acids increased by a factor of 2 to 4 for acetate and propionate according to the stools and by a factor of 2 to 6 for butyrate. The bacterial metabolites, although volatile are present in the lyophilisates. The increase in the concentration thereof would be a consequence of the lyophilisation which results in a concentration of the metabolites (Figure 9).

2.2.2. Detection of the anti-*Clostridium difficile* effect in the lyophilisates

The anti-*Clostridium difficile* effect of the native stools as well as lyophilisates was analysed *in vitro* in the presence of 2 strains of *Clostridium difficile*, a toxinogenic strain (strain 630) and the other non-toxinogenic (strain PCD1). When the strains are inhibited in their growth, a pale circle appears around the inoculation zone: this is the inhibition circle.

Various controls, known for their anti-*Clostridium* effects were systematically used as positive controls. Acetic acid (AA) as well as lactic acid (AL) were thus used at different concentrations (300, 400, 500 mM). In each dish, an inhibition circle was observed in the presence of acetic acid and/or lactic acid.

Pure strains were used as a control, namely, a strain of *Lactobacillus* and a strain of *Bifidobacterium* (CB) which do not result in the appearance of inhibition circles.

In the presence of native stools (S or SN) as in the presence of stools that were frozen at -80°C for 72h (T1, T2 and T3), no inhibition circle was observed while an inhibition circle is visible in the presence of the lyophilisates (Figure 10).

The results of the various experiments showed the presence of an inhibition zone for acetic acid and lactic acid, as well as for the lyophilisates of all the stools analysed. The inhibition capacity of the development *in vitro* of *C. difficile* by a stool is therefore potentiated by lyophilisation.

All of the results presented in example 2 confirm the stability and the viability of the microbiota within the lyophilisate over a period of at least 6 months and shows the maintaining of the functional capacities of bacteria within the lyophilisate with in particular a preservation of bacterial metabolites of interest and an anti-*Clostridium difficile* effect in a study model *in vitro*.

EXAMPLE 3

1. Equipment and Methods

1.1. Lyophilisates of stools

Four stools coming from healthy donors were treated in application of the method described in example 1, in order to obtain four faecal lyophilisates: S4, S5, S6 and S7, S7 represent an additional faecal sample with respect to example 2.

1.2. Choice of excipients

Diluents

The choice of diluents was based on their inert nature and in particular on their absence of specific effects at the intestinal level.

The diluents selected are a mineral excipient, dicalcium phosphate (anhydrous Emcompress®, JRS Pharma, Rosenberg, Germany), and nebulised maltodextrins (Emdex®, JRS Pharma, Rosenberg, Germany), with this last type of excipient already part of the composition of the lyophilisate. The density and the flow time of these two pure excipients are respectively, 0.79 g/mL, and 3 seconds 18/100 g for anhydrous Emcompress®, and 0.68 g/mL, and 6 seconds 03/100 g for Emdex®.

10 Lubricants

Three flow lubricants were selected: talc (Cooper, France), a hydrophilic colloidal silica (Aerosil® 200 Pharma, Kraemer & Martin GmbH, St-Augustin, Germany), and a hydrophobic colloidal silica [Aerosil® R972, Degussa-Hüls, Courbevoie, France].

15 1.3. Measurement of the flow time and mixture of the lyophilisates with the excipients

This test was conducted according to the monograph 2.9.16 of the European Pharmacopoeia, on the lyophilisates alone, then after the addition of a flow lubricant used at 0.5%, and if needed a diluent tested in the weight ratios 75/25, 50/50 and 25/75. The mixtures were carried out manually.

The density of the final mixtures having good fluidity was measured over all the mixtures, then converted to 1 mL.

25 1.4. Capsule filling with the mixtures having a good flow

Capsules No. 00, combining a substantial capacity of 0.95 mL and a size compatible with a relatively easy ingestion (23.3 mm long) were chosen. They were filled manually on a capsule filler with the mixtures having a good flow.

30 2. Results

2.1. Study of the flow and density characteristics of the lyophilisates

Table 1 groups together the results of the tests conducted on the lyophilisates.

Table 1. Flow of raw faecal lyophilisate and in mixtures with a flow lubricant and/or a diluent, and density of the final mixtures

	MIXTURES TESTED	WEIGHT RATIO	FLOW (average of 3 measurements for x g)	DENSITY
S4	Raw faecal lyophilisate 1	-	Infinite	Not determined
	Faecal lyophilisate/talc	99.5/0.5	Infinite	
	Faecal lyophilisate-talc*/Emdex®	75/25	Infinite	
		50/50	4s16 (tapping)	
		25/75	0s69 for 13.70 g (0s69/0s68/0s71)	13.70 g/26 ml (0.53 g/mL)
S5	Raw faecal lyophilisate	-	Infinite	Not determined
	Faecal lyophilisate/Aerosil® 200	99.5/0.5	Infinite	
	Faecal lyophilisate-Aerosil® 200*/anhydrous Emcompress®	75/25	0s84/0s37/Infinite (tapping)	
		50/50	0s53/0s31/Infinite	
		25/75	0s57 for 10.86 g (0s56/0s48/0s68)	10.86 g/17 mL (0.64 g/mL)
S6	Raw faecal lyophilisate	-	Infinite	Not determined
	Faecal lyophilisate/Aerosil® R972	99.5/0.5	0s30 pour 1.466 g (0s35/0s25/0s31)	1.466 g/4.5 mL (0.33 g/mL)
S7	Raw faecal lyophilisate	-	Infinite	Not determined
	Lyophilisate/Aerosil® R972	99.5/0.5	0s26 for 3.296 g (0s25/0s25/0s27)	3.296 g/7 mL (0.47 g/mL)

*at a weight ratio 99.5/0.5

5

None of the raw faecal lyophilisates tested alone has any flow property. It is therefore impossible to put them in capsules as is.

Despite their role as a flow lubricant, talc and Aerosil® 200 used at 0.5% did not make it possible to improve the fluidity of the lyophilisates (S4 and S5).

10

The Emdex® and anhydrous Emcompress® diluents are effective in improving the flow. The improvement occurs from the ratio 50/50 for the lyophilisate S4-talc/Emdex® mixture, and from the ratio 75/25 for the lyophilisate S5-Aerosil® 200/anhydrous Emcompress® mixture. At the ratio 25/75 between lyophilisate and diluent, the mixture becomes fluid with a flow time of 0s69 for 13.70 g (S4) and 0s57 for 10.86 g (S5).

Finally, the flow lubricant Aerosil® R972, used alone at 0.5%, allowed for a very clear improvement in the fluidity of the lyophilisates S6 (0s30 for 1.466 g) and S7 (0s26 for 3.296 g).

The density of the mixtures varies from 0.53 to 0.64 g/mL for the "lyophilisate/flow lubricant/diluent" mixtures, and from 0.33 to 0.47 g/mL for the "lyophilisate/flow lubricant" mixtures.

2.2. Capsule filling with the mixtures

Table 2 shows the number of capsules prepared for each sample and the mass of the lyophilisate incorporated per capsule. The theoretical weight of the fresh material used initially is reported in the last column.

Table 2. Characteristics of the capsules prepared from lyophilised samples

	NUMBER OF CAPSULE S PREPARE D	MASS OF MIXTURE/CAPSU LE	MASS OF LYOPHILISATE/CAPSU LE	THEORETICA L MASS OF FRESH MATERIAL IN n CAPSULES
S4- talc/Emdex®	20	500 mg	125 mg	1754 mg
S5-Aerosil® 200/anhydro us Emcompress®	18	603 mg	151 mg	3000 mg
S6-Aerosil® R972	5	293 mg	293 mg	1792 mg
S7-Aerosil® R972	7	471 mg	471 mg	5000 mg

The case of the samples S6 and S7 is the most favourable because the dilution of the lyophilisate is negligible due to the adding of only 0.5% of flow lubricant. However, a rather great variability is observed between the two samples which, under the same volume, can represent 293 mg (S6) or 471 mg (S7) of dry matter. This is due to the difference in the density between the two samples.

Regarding the samples S4 and S5, the dilution of the lyophilisate is more substantial due to the adding of 75% of diluent, in addition to the 0.5% of flow lubricant. Under the

same volume, the mass of lyophilisate varies from 125 mg (S4) to 151 mg (S5) of dry matter, in relation with the density of each sample.

This study shows that lyophilisates of faecal microbiota reduced to a powder do not have any property of fluidity and therefore cannot be put into capsules alone.

- 5 On the other hand, it is possible to improve the flow properties of the lyophilisates by mixing, either with 75% diluent (Emdex® maltodextrins, anhydrous dicalcium phosphate anhydrous Emcompress®), or with 0.5% of flow lubricant (hydrophobic colloidal silica, Aerosil® R972).

BIBLIOGRAPHY

Allen-Vercoe E, Petrof EO. Artificial stool transplantation: progress towards a safer, more effective and acceptable alternative. *Expert Rev Gastroenterol Hepatol* 2013;7:291-3.

ANSM. Report. La transplantation de microbiote fécal et son encadrement dans les essais cliniques, March 2014, updated June 2015.

Béal C and Fonseca F. Freezing of probiotic bacteria. P. 179-212. *Advances in probiotic technology*. Eds Foerst P. and Sativarangkna C. 2015, CRC Press, Boca Raton, USA.

Borody T, Fischer M, Mitchell S, Campbell J. Fecal microbiota transplantation in gastrointestinal disease: 2015 update and the road ahead. *Expert Rev Gastroenterol Hepatol*. 2015; 9:1379-91.

Carvalho A.S., Silva J., Ho P., Texeira P., Malcata F.X. and Gibbs P. Relevant factors for the preparation of freeze-dried lactic acid bacteria. *Int. Dairy J* 2004, 14: 835-47.

Debast SB, Bauer MP, Kuijper EJ. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. *Clin Microbiol Infect* 2014; 20 Suppl 2:1-26.

Hamilton MJ, Weingarden AR, Sadowsky MJ, Khoruts A. Standardized frozen preparation for transplantation of fecal microbiota for recurrent *Clostridium difficile* infection. *Am J Gastroenterol* 2012; 107:761-7.

Kapel N, Thomas M, Corcos O, Mayeur C, Barbot-Trystram L, Bouhnik Y, Joly F, Practical Implementation of faecal transplantation. *Clinical Microbiology and Infection* 2014; 20:1098-105.

Kelly CR, Kahn S, Kashyap P, Laine L, Rubin D, Atreja A, Moore T, Wu G. Gastroenterology. 2015 Jul; 149(1): 223-37.

5 Le Hir A, Chaumeil JC, Brossard D, Charrueau C, Crauste-Mancier S. Pharmacie galénique, bonnes pratiques de fabrication des médicaments. Elsevier Masson SAS, Issy-les-Moulineaux, France, 10th edition, 2016.

10 Lee CH, Steiner T, Petrof EO, Smieja M, Roscoe D, Nematallah A, Weese JS, Collins S, Moayyedi P, Crowther M, Ropeleski MJ, Jayaratne P, Higgins D, Li Y, Rau NV, Kim PT. Frozen vs Fresh Fecal Microbiota Transplantation and Clinical Resolution of Diarrhea in Patients With Recurrent *Clostridium difficile* Infection: A Randomized Clinical Trial. JAMA. 2016; 315:142-149

15 Ott SJ, Waetzig GH, Rehman A, Moltzau-Anderson J, Bharti R, Grasis JA, Cassidy L, Tholey A, Fickenscher H, Seegert D, Rosenstiel P, Schreiber S. Efficacy of Sterile Fecal Filtrate Transfer for Treating Patients With *Clostridium difficile* Infection. Gastroenterology. 2016 Nov 17. pii: S0016-5085(16)35354-9. doi: 10.1053/j.gastro.2016.11.010.

20 European Pharmacopeia 8th edition, 2016. Monograph 2.9.16. Flow. EDQM European Directory for the Quality of Medicines and Healthcare. <https://www.edqm.eu/fr/Pharmacopee-europeenne-8eme-Edition-1563.html>

25 Rougé C, Goldenberg O, Ferraris L, Berger B, Rochat F, Legrand A, Göbel UB, Vodovar M, Voyer M, Rozé JC, Darmaun D, Piloquet H, Butel MI, De La Cochetière MF. Investigation of the intestinal microbiota in preterm infants using different methods. Anaerobe 2010; 16:362-370.

30 Sokol H, Galperine T, Kapel N, Bourlioux P, Seksik P, Barbut F, Scanzi J, Chast F, Batista R, Joly F, Joly AC, Collignon A, Guery B, Beaugier L, for the Groupe Français de Transplantation Fécale (GFTF). Transplantation de microbiote fécal dans le cadre des infections à *Clostridium difficile* récidivantes: recommandations pour la pratique clinique courante. Hepato Gastro 201; 22:278890.

Surawicz CM, Brandt LJ, Binion DG, et al. Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. Am J Gastroenterol 2013; 108:478-98.

5 Van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller J. Duodenal infusion of donor feces for recurrent *Clostridium difficile*. N Engl J Med 2013; 368:407-15.

10 Vrieze A, de Groot PF, Kootte RS, Knaapen M, van Nood E, Nieuwdorp M. Fecal transplant: a safe and sustainable clinical therapy for restoring intestinal microbial balance in human disease. Best Pract Res Clin Gastroenterol 2013; 27:127-37.

15 Youngster I, Sauk J, Pindar C, Wilson RG, Kaplan JL, Smith MB, Alm EJ, Gevers D, Russell GH, Hohmann EL. Fecal microbiota transplant for relapsing *Clostridium difficile* infection using a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. Clin Infect Dis 2014a; 58:1515-22

20 Youngster I, Russell GH, Ziv-Baran T, Sauk J, Hohmann EL. Oral, capsulized, frozen fecal microbiota transplantation for relapsing *Clostridium difficile* infection. JAMA. 2014b; 312:1772-8.

25 Youngster I, Mahabamunuge J, Systrom HK, Sauk J, Khalili H, Levin J, Kaplan JL, Hohmann EL. Oral, frozen fecal microbiota transplant (FMT) capsules for recurrent *Clostridium difficile* infection. BMC Med. 2016 Sep 9; 14(1):134. doi: 10.1186/s12916-016-0680-9.

Patentkrav

1. Anvendelse af en blanding af kryoprotektorer til konservering af en mikrobiota i dens økosystem i lyofiliseret form, **kendetegnet ved, at** blandingen af
5 kryoprotektorer omfatter maltodextrin (M) og trehalose (T) i et vægtforhold M/T mellem 35:65 og 45:55, idet mikrobiotaen i sit økosystem er tarmmikrobiota, fækal mikrobiota eller vaginal mikrobiota.
2. Anvendelse ifølge krav 1, ved hvilken mikrobiotaen i sit økosystem består af
10 afføring.
3. Lyofiliseret sammensætning omfattende en mikrobiota i dens økosystem og en blanding af kryoprotektorer, **kendetegnet ved, at** blandingen af kryoprotektorer omfatter maltodextrin og trehalose, der anvendes i et vægtforhold M/T mellem
15 35:65 og 45:55, idet mikrobiotaen i sit økosystem er tarmmikrobiota, fækal mikrobiota eller vaginal mikrobiota.
4. Lyofiliseret sammensætning ifølge krav 3, i hvilken (i) mikrobiotaen i sit økosystem (MDSE) består af afføring, (ii) vægtmængden af afføring udgør 15 til
20 35% af den lyofiliserede sammensætning, og vægtmængden af blandingen af kryoprotektorer udgør 85 til 65% af den lyofiliserede sammensætning, og (iii) maltodextrin (M) og trehalose (T) anvendes i et vægtforhold M/T på 40:60.
5. Gastroresistent gelatinekapsel omfattende en lyofiliseret sammensætning som
25 defineret i et af kravene 3 eller 4.
6. Gastroresistent gelatinekapsel ifølge krav 5, **kendetegnet ved, at** den lyofiliserede sammensætning er blandet med mindst et flydehjælpemiddel.
- 30 7. Lyofiliseret sammensætning ifølge et af kravene 3 eller 4 eller gastroresistent gelatinekapsel ifølge krav 5 eller 6 til anvendelse ved behandling af en sygdom eller en lidelse i forbindelse med en uligevægt i tarmmikrobiotaen valgt blandt colitis med *Clostridium difficile* og dens recidiver, kroniske inflammationssygdomme i tarmen, såsom morbus Chron og colitis ulcerosa, colon
35 irritabile, bæren af multiresistente bakterier, forstoppelse, rejsediarre, infektioner

i fordøjelsesstymet, autoimmunsygdomme, diverticulose, diabetes type 1 og type 2, stofskiftesyndrom, obesitas, arthritis rheumatoides, spondylitis ankylosus og sakroilitis, kronisk træthedssyndrom, halitose, allergi, akne, søvnløshed, depressioner og neuropsykiatriske sygdomme, såsom Parkinsons sygdom og

5 sygdommene fra autisme-spektret.

8. Lyofiliseret sammensætning eller gastroresistent gelatinekapsel til anvendelse ifølge krav 7 i tilslutning til en oral antibiotikabehandling i tilfælde af recidiver af infektioner med *Clostridium difficile*.

10

9. Fremgangsmåde til fremstilling af en gastroresistent gelatinekapsel som defineret i krav 5 eller 6, omfattende følgende trin:

- a. opsamling af en mikrobiota i dens økosystem;
 - b. fortynding af mikrobiotaen i dens økosystem med en blanding af
 - 15 kryoprotektorer, der omfatter maltodextrin og trehalose i et vægtforhold M/T mellem 35:65 og 45:65;
 - c. lyofilisering af den i trin b opnåede blanding;
 - d. pulverisering af det i trin c opnåede lyofilisat;
 - e. eventuel tilsætning af et eller flere hjælpestoffer, der letter lyofilisatets
 - 20 flydning;
 - f. fyldning af den gastroresistente gelatinekapsel med den lyofiliserede blanding;
 - g. lukning af gelatinekapslen,
- idet mikrobiotaen i sit økosystem er tarmmikrobiota, fækal mikrobiota eller
- 25 vaginal mikrobiota.

10. Fremgangsmåde ifølge krav 9, ved hvilken mikrobiotaen i sit økosystem består af afføring, og blandingen af kryoprotektorer består af en blanding af maltodextrin (M) og trehalose (T) i et vægtforhold M/T på 40:60.

30

1 / 6

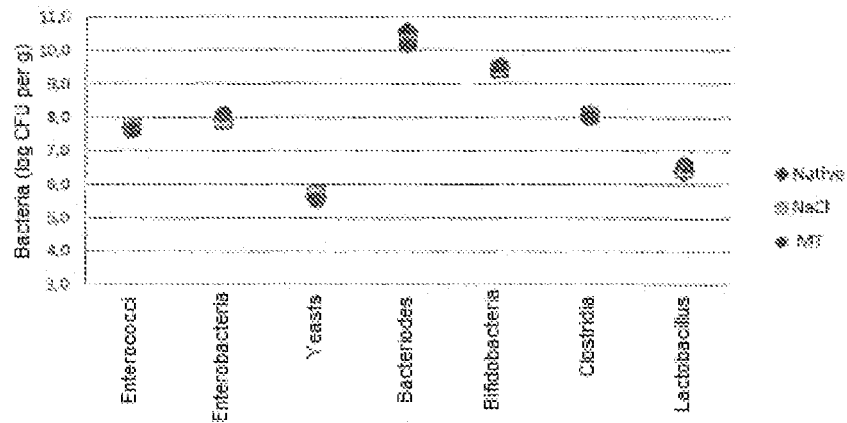


Figure 1

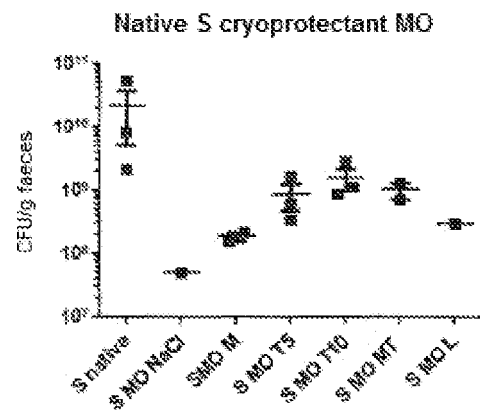


Figure 2

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preservation of stools in cryoprotectant: bacterial load

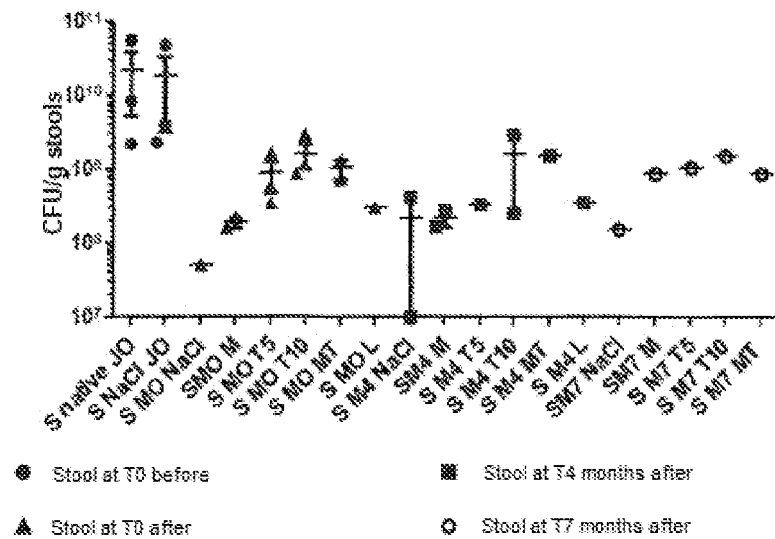


Figure 3

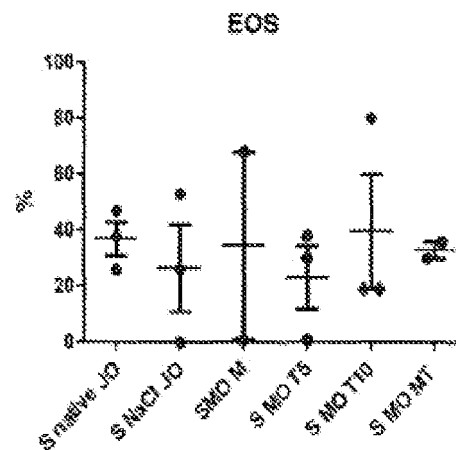


Figure 4

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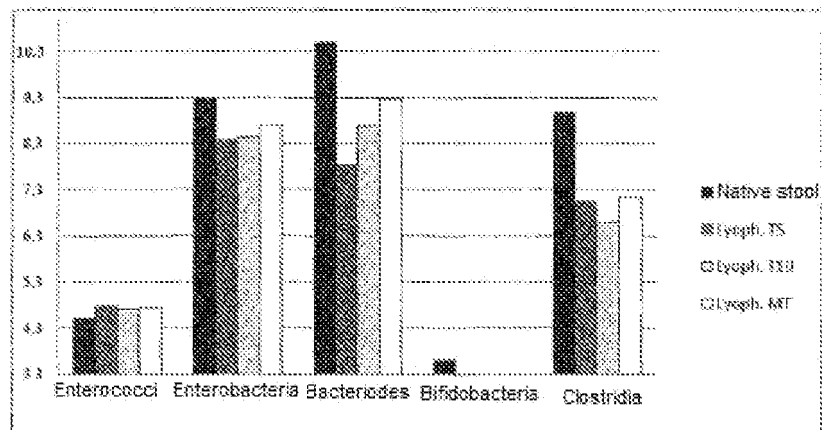


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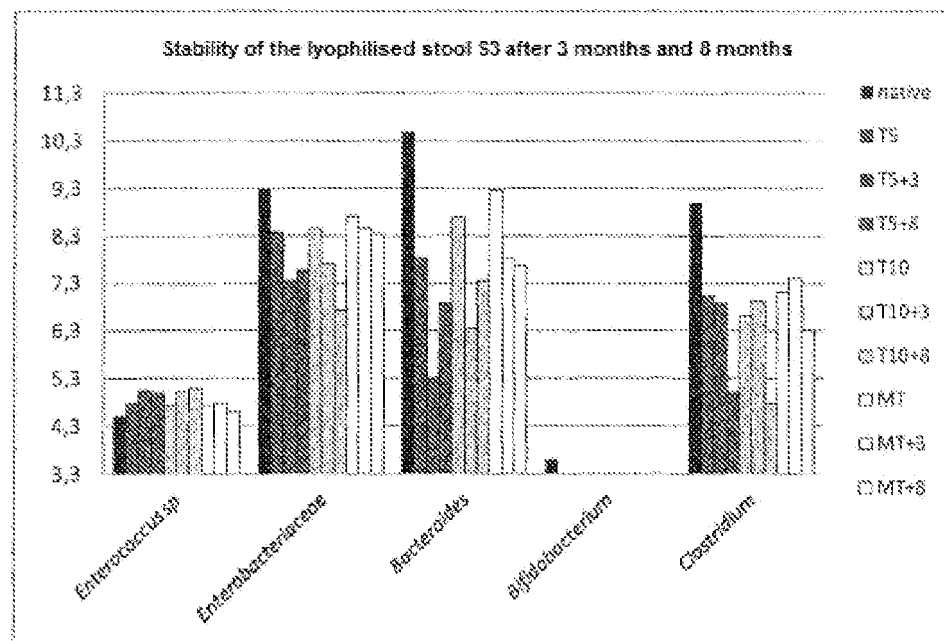


Figure 6

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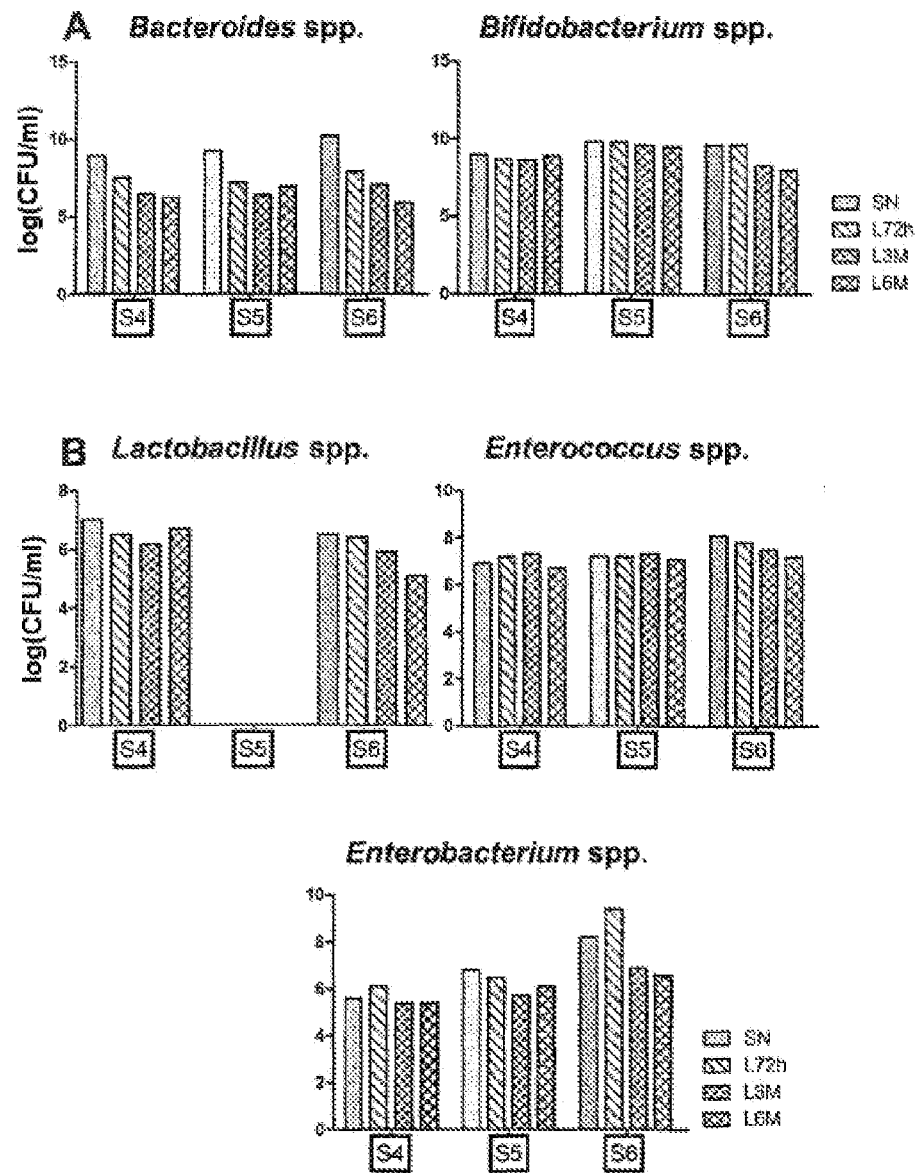


Figure 7

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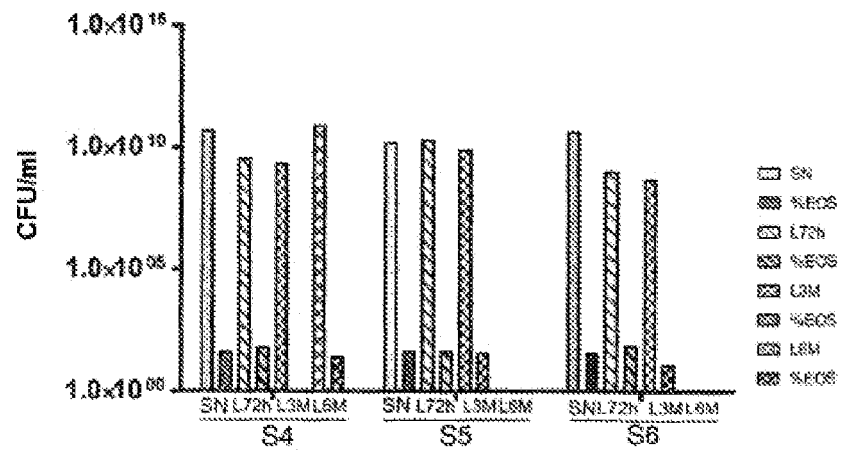


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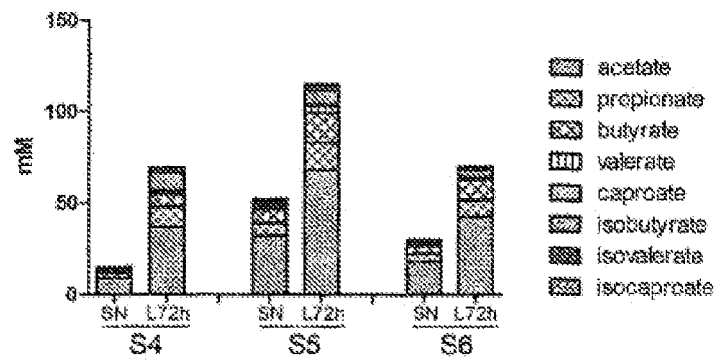


Figure 9

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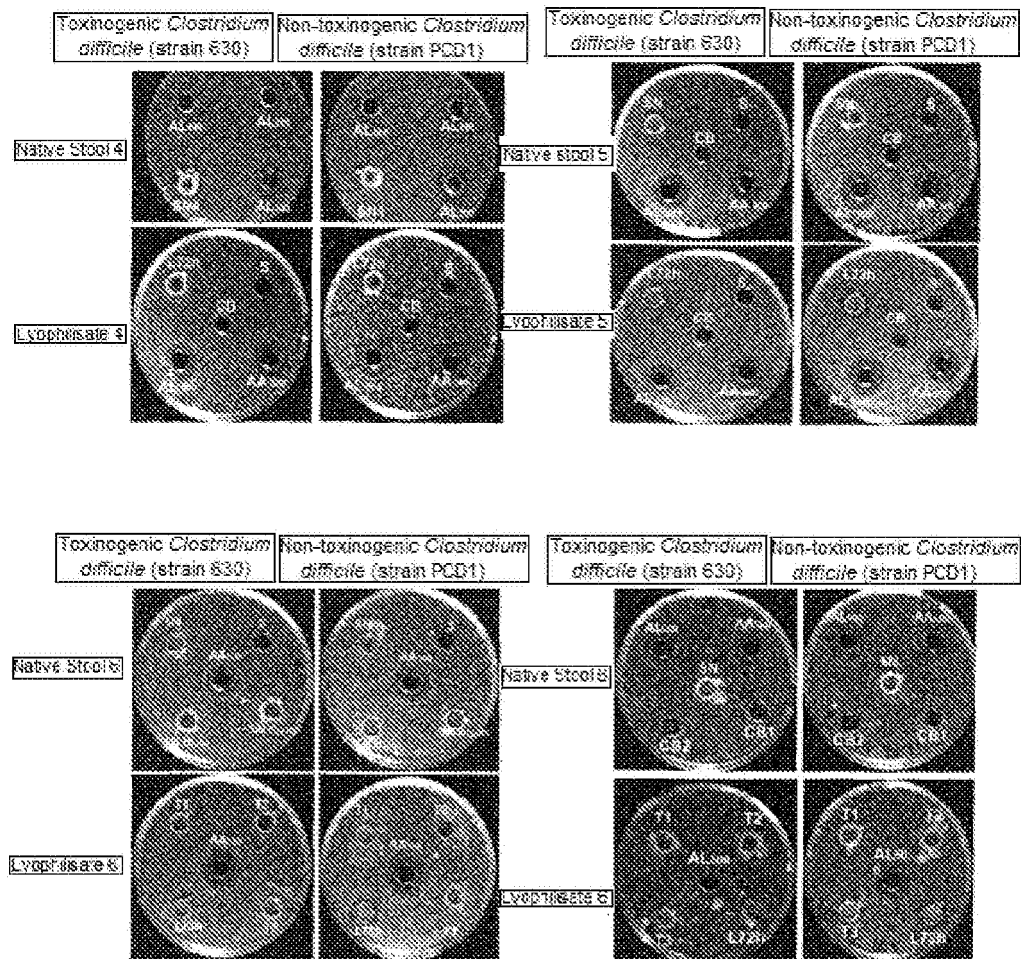


Figure 10