

COMPOSITIONS COMPRISING HUMAN MILK OLIGOSACCHARIDES AND INACTIVATED CELLS OF A MICROORGANISM

FIELD OF THE INVENTION

The present invention relates to a combination of a human milk oligosaccharides (HMOs) and
5 inanimate cells of a microorganism, compositions comprising the combination and the use of
the combination or the compositions to promote γ -aminobutyric acid or lactate production in the
gut of a mammal.

BACKGROUND OF THE INVENTION

Lactic acid and γ -aminobutyric acid (GABA) production in the gut are both influenced by the
10 activity of specific gut microbiota, contribute to gut health, interact with the gut-brain axis, and
have potential therapeutic applications.

Although it has been traditionally regarded as an intermediate of carbon metabolism and major
component of fermented dairy products contributing to organoleptic and antimicrobial properties
of food, there is evidence gathered in recent years that lactate has bioactive properties that may
15 be responsible of broader properties of functional foods. Lactate can regulate critical functions
of several key players of the immune system such as macrophages and dendritic cells, being
able to modulate inflammatory activation of epithelial cells as well. Intraluminal levels of lactate
derived from fermentative metabolism of lactobacilli have been shown to modulate inflammatory
environment in intestinal mucosa. Moreover, besides immunomodulation, it has been shown
20 that luminal lactate stimulated enterocyte proliferation in a murine model of hunger-feedback,
contributing to maintain intestinal barrier function. Lactate has also been shown to inhibit the
growth of some pathogenic bacteria, including *Escherichia coli*, and can also reach high
concentrations in the gut of healthy infants, reflecting the dominance of L-lactate-producing
bifidobacteria. In addition, lactate can decrease gas production from fermentation of
25 carbohydrates.

Lactate production is widespread among human gut microbes. Many types of bacteria produce
lactic acid, among others many different gut bacteria belonging to the dominant phyla of
Bacteroidetes, Firmicutes (including *Lactobacillus* and *Streptococcus* species within the order
Lactobacillales, which generate principally lactate as an acidic fermentation end product),
30 Proteobacteria and *Bifidobacterium* species within the phylum Actinobacteria.

Lactate can be utilized by particular species of the *Firmicutes* phylum that are able to produce
the short chain fatty acids (SCFAs) butyrate or propionate from lactate in the gut. Since these
SCFAs are known to exert a number of positive effects for the host, lactate utilization can be

considered an indirectly beneficial consequence of lactate production by other members of the gut microbiota.

GABA is an important neurotransmitter that plays a crucial role in brain development and function. It is involved in regulating neuronal activity and is believed to have a calming effect on the brain. Studies have suggested that GABA may have a number of health benefits including children, including improving cognitive function, reducing anxiety and stress, and promoting healthy sleep patterns. GABA has also been shown to have a positive impact on certain developmental disorders, such as autism and attention deficit hyperactivity disorder (ADHD).

GABA can also be found in certain fermented foods, such as kimchi, yogurt, and kefir, and may be produced by some probiotic lactobacilli strains. GABA has also been shown to have potential benefits for gut health. For example, GABA may support gut barrier function and tight junctions and thereby contribute to ameliorating the effects caused by pathogens on the gut barrier (see e.g. Kaur et al. *Microbial Cell Factories* **22**:256 (2023)). Furthermore, GABA may help to reduce inflammation in the gut, e.g. by reducing production of proinflammatory cytokines, such as TNF- α , IL6 and IL8, through downregulation of p38 mitogen-activated protein kinase (MAPK), which could be beneficial in treating or reducing symptoms for individuals with inflammatory bowel disease (IBD) or other gut-inflammatory conditions (see e.g. Aggarwal et al. *J. Neurogastroenterol. Motil.* **24**, 422 (2018)). Additionally, GABA may help to regulate gut motility, which could help to alleviate symptoms of constipation or diarrhoea (Kerr and Ong: *GABA and Gut Motility*. In: Erdö, S.L. (eds) *GABA Outside the CNS*. Springer, Berlin, Heidelberg, pp 29–44, 1992)).

Gut barrier function refers to the ability of the gut to prevent the leakage of harmful substances, such as toxins, bacteria, and undigested food particles, from the gut lumen into the bloodstream. The gut barrier is composed of several different components, including the gut epithelium, which is the layer of cells that lines the gut, as well as the mucus layer, which acts as a physical barrier to prevent direct contact between the gut epithelium and the gut lumen. The gut barrier also includes the tight junctions between gut epithelial cells, which help to regulate the passage of substances across the gut epithelium. When the gut barrier is compromised, harmful substances can leak into the bloodstream, and potentially trigger inflammation and immune responses. Several factors can contribute to the breakdown of the gut barrier, including poor diet, stress, infections, and medications such as nonsteroidal anti-inflammatory drugs (NSAIDs).

The mucosal barrier is part of the gut barrier function and is a layer of mucus that lines the gastrointestinal tract and protects the body from harmful microorganisms and substances. The mucosal barrier function is not fully developed at birth, and the mucosal barrier continues to

mature during the first year of life. In infants, this barrier is thinner and more permeable than in adults, which can make them more susceptible to infections and food allergies. Human milk oligosaccharides (HMOs) are a heterogeneous mixture of soluble glycans found in human milk. They are the third most abundant solid component after lactose and lipids in human milk and
5 are present in concentrations of 5-25 g/l (Bode: *Human milk oligosaccharides and their beneficial effects*, in: Handbook of dietary and nutritional aspects of human breast milk (Zibadi et al., eds.), pp. 515-31, Wageningen Academic Publishers (2013)). The structure of the HMOs is similar to the O-glycans found in mucus and the N-glycans found on human cells. HMOs are resistant to enzymatic hydrolysis in the small intestine and are thus largely undigested and
10 unabsorbed. The majority of HMOs that reach the colon serve as substrates to shape the gut ecosystem by selectively stimulating the growth of specific bacteria. HMOs are believed to substantially modulate the infant gut microbiota and play a decisive role in the differences in the microbiota of formula-fed and breast-fed infants.

Non-viable microorganisms or microbial cell extracts, mainly heat-killed (including tyndallized)
15 bacteria (lactic acid bacteria and bifidobacteria), are able to produce beneficial effects. For example, dead cells of *Lactobacillus* strains are useful to produce a psychobiotic effect in a human (WO 2019/180263), in stimulating the growth of bifidobacteria in the human gut (WO 2021/219846) or to treat or prevent an allergic disease (EP-A-2796142). However, as being inanimate, they are not supposed to produce lactic acid or GABA.

20 There is a need, therefore, for means, preferably orally or enterally administered means, more preferably dietetic means, to promote lactic acid and/or GABA production in the gut of mammals.

SUMMARY OF THE INVENTION

The first aspect of this invention relates to a combination of a human milk oligosaccharide
25 (HMO) and inactivated cells of a microorganism. The combination, in the present context, means that it comprises a HMO and inactivated cells of a microorganism, preferably consists essentially of or consists of one or more HMOs and dead/inactivated cells of one or more microorganisms optionally with or in its/their concentrated or dried fermentation medium from which the microorganism is derived and the medium components therein. If the combination
30 above contains the dried fermentation medium of the inactivated cells, it may optionally further contain lactose as carrier facilitating the drying process. Preferably, the combination consists or consists essentially of one or more HMOs and dead/inactivated cells of one or more microorganisms in or with its/their dried fermentation medium comprising medium components and optionally lactose.

The second aspect of the invention relates to a synthetic composition comprising the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism as defined in the first aspect. The synthetic composition is, in one embodiment, a nutritional composition that confers a nutritional (that is non-therapeutic) benefit to the mammal consuming it. The synthetic composition, in other embodiment, is a pharmaceutical composition that restores the organism of a mammal from a pathological to its original condition. The synthetic composition comprising the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism as defined in the first aspect contains nutritionally or pharmaceutically acceptable ingredients other than the components of the combination.

5 The third aspect of the invention relates to a combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, for use in a therapy or as a medicament, preferably for use in treating conditions of a mammal wherein enhanced level of lactate and/or GABA production in the gut of the mammal contributes to treat pathological conditions or clinical situations.

10 The fourth aspect of the invention relates to a synthetic composition comprising the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism as defined in the second aspect of the invention for use in therapy or as a medicament, preferably for use in treating conditions of a mammal wherein enhanced level of lactate and/or GABA production in the gut of the mammal contributes to treat pathological conditions or clinical situations.

15 The fifth aspect of the invention relates to a method for promoting or increasing the lactate production in the gut of a mammal, the method comprising orally or enterally administering to, or providing for consumption by, the mammal an effective amount of a combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, the combination optionally being comprised in a synthetic composition, as defined in the second aspect of the invention.

20 The sixth aspect of the invention relates to a method for promoting or increasing the GABA production in the gut of a mammal, the method comprising orally or enterally administering to, or providing for consumption by, the mammal an effective amount of a combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, the combination optionally being comprised in a synthetic composition, as defined in the second aspect of the invention.

25 The seventh aspect of the invention relates to a non-therapeutic use of the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, for promoting or enhancing the lactate production in the gut of a mammal.

The eighth aspect of the invention relates to a non-therapeutic use of the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, for promoting or enhancing the GABA production in the gut of a mammal.

- 5 The ninth aspect of the invention relates to a non-therapeutic use of the synthetic composition comprising the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the second aspect of the invention, for promoting or enhancing the lactate production in the gut of a mammal.

- The tenth aspect of the invention relates to a non-therapeutic use of the synthetic composition
10 comprising the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the second aspect of the invention, for promoting or enhancing the GABA production in the gut of a mammal.

- In all aspects of the invention, the HMO can be a neutral HMO or an acidic HMO, preferably a neutral HMO. The neutral HMO can be one or more fucosylated HMOs or one or more non-
15 fucosylated HMOs. Preferably, the HMO is 2'-FL, 3-FL, DFL, LNT, LNnT, LNFP-I, LNDFH-I or a mixture thereof. Also preferably, the HMO comprises 2'-FL, LNnT, 3'-SL, 6'-SL or any combination thereof.

Also, in all aspects of the invention, the microorganism is preferably a *Lactobacillus*, more preferably a mixture of *L. fermentum* and *L. delbrueckii*.

- 20 In all aspects of the invention, the mammal is preferably human, more preferably a non-infant human.

DETAILED DESCRIPTION OF THE INVENTION

Herein, the following terms have the following meanings:

"About" or "around" means +/- 5 %.

- 25 "Billion" is one thousand million, that is 10^9 (ten to the ninth power).

- "Effective amount" means an amount of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, optionally in a suitable composition, that provides these ingredients in a sufficient amount to render a desired health outcome in a human. An effective amount can be administered or provided for consumption in one or more doses to achieve the desired health
30 outcome.

"Enteral administration" means any conventional form for delivery of a composition to a human that causes the deposition of the composition in the gastrointestinal tract (including the

stomach). Methods of enteral administration include feeding through a naso-gastric tube or jejunum tube, oral, sublingual and rectal.

"Human milk oligosaccharide" or "HMO" means a complex carbohydrate found in human breast milk (Urashima et al.: *Milk Oligosaccharides*. Nova Science Publisher (2011); Chen *Adv.*

5 *Carbohydr. Chem. Biochem.* 72, 113 (2015)). The HMOs have a core structure comprising a lactose unit at the reducing end that can be elongated by one or more β -N-acetyl-lactosaminyl and/or one or β -more lacto-N-biosyl units, and which core structure can be substituted by an α -L-fucopyranosyl and/or an α -N-acetyl-neuraminyl (sialyl) moiety. In this regard, the non-acidic (or neutral) HMOs are devoid of a sialyl residue, and the acidic HMOs have at least one sialyl
10 residue in their structure. The non-acidic (or neutral) HMOs can be fucosylated or non-fucosylated. Examples of such neutral non-fucosylated HMOs include lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-neohexaose (LNnH), para-lacto-N-neohexaose (pLNnH), para-lacto-N-hexaose (pLNH) and lacto-N-hexaose (LNH). Examples of neutral fucosylated HMOs include 2'-fucosyllactose (2'-FL), lacto-N-fucopentaose I (LNFP-I), lacto-N-difucohexaose
15 I (LNDFH-I), 3-fucosyllactose (3-FL), difucosyllactose (DFL), lacto-N-fucopentaose II (LNFP-II), lacto-N-fucopentaose III (LNFP-III), lacto-N-difucohexaose III (LNDFH-III), fucosyl-lacto-N-hexaose II (FLNH-II), lacto-N-fucopentaose V (LNFP-V), lacto-N-fucopentaose VI (LNFP-VI), lacto-N-difucohexaose II (LNDFH-II), fucosyl-lacto-N-hexaose I (FLNH-I), fucosyl-para-lacto-N-hexaose I (FpLNH-I), fucosyl-para-lacto-N-neohexaose II (F-pLNnH II) and fucosyl-lacto-N-
20 neohexaose (FLNnH). Examples of acidic HMOs include 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), 3-fucosyl-3'-sialyllactose (FSL), LST a, fucosyl-LST a (FLST a), LST b, fucosyl-LST b (FLST b), LST c, fucosyl-LST c (FLST c), sialyl-LNH (SLNH), sialyl-lacto-N-hexaose (SLNH), sialyl-lacto-N-neohexaose I (SLNH-I), sialyl-lacto-N-neohexaose II (SLNH-II) and disialyl-lacto-N-tetraose (DSLNT).

25 "Inactivated cells of a microorganism" preferably means inanimate microorganism that has been made deliberately lifeless (dead or killed), therefore is not able to colonize under appropriate conditions, for example in the host's gut. The degree of inactivation is at least 90 %, preferably at least 95 %, more preferably at least 98 %, even more preferably at least 99 %, advantageously substantially all cell of the microorganism is dead or killed, more
30 advantageously the entire population of the microorganism is dead or killed.

"Lactate" refers to lactic acid and they are used interchangeably.

"Microbiota", "microflora" and "microbiome" mean a community of living microorganisms that typically inhabits a bodily organ or part, particularly the gastro-intestinal organs of non-infant humans. The most dominant members of the gastrointestinal microbiota include
35 microorganisms of the phyla of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria,

Synergistetes, Verrucomicrobia, Fusobacteria, and Euryarchaeota; at genus level *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Roseburia*, *Alistipes*, *Collinsella*, *Blautia*, *Coprococcus*, *Ruminococcus*, *Eubacterium* and *Dorea*; at species level *Bacteroides uniformis*, *Alistipes putredinis*, *Parabacteroides merdae*, *Ruminococcus bromii*, *Dorea longicatena*, *Bacteroides* 5 *caccae*, *Bacteroides thetaiotaomicron*, *Eubacterium hallii*, *Ruminococcus torques*, *Faecalibacterium prausnitzii*, *Ruminococcus lactaris*, *Collinsella aerofaciens*, *Dorea formicigenerans*, *Bacteroides vulgatus* and *Roseburia intestinalis*. The gastrointestinal microbiota includes the mucosa-associated microbiota, which is located in or attached to the mucus layer covering the epithelium of the gastrointestinal tract, and luminal-associated 10 microbiota, which is found in the lumen of the gastrointestinal tract.

“Non-infant human” or “non-infant” means a human of 3 years of age and older. A non-infant human can be a child, a teenager, an adult or an elderly person.

“Non-therapeutical use” means - as opposed to the purpose of therapy which is to restore the organism from a pathological to its original condition, or to prevent pathology in the first place – 15 an improvement of performance of the organism in a normal state. Therefore, the purpose of the non-therapeutical use is to enhance performance and perception of well-being in a healthy state of a mammal/individual or at least in a mammal/individual that is not likely to develop a pathological state.

“Nutraceutical composition” refers to a formulation that combines nutrients and/or bioactive 20 compounds, designed to provide health benefits beyond basic nutrition. These compositions can be in the form of dietary supplements, functional foods, or beverages that aim to prevent or treat diseases, enhance health, and improve overall well-being.

“Oral administration” means any conventional form for the delivery of a composition to a human through the mouth. Accordingly, oral administration is a form of enteral administration.

25 “Probiotic microorganism” means live microorganism (beneficial bacterium or yeast) which, when administered in adequate amounts, confers a health benefit on the host, generally by improving or restoring the gut microbiota.

“Synthetic composition” means a composition which is artificially prepared and preferably 30 means a composition containing at least one compound that is produced *ex vivo* chemically and/or biologically, e.g. by means of chemical reaction, enzymatic reaction or recombinantly. In some embodiments, the synthetic compositions may comprise one or more nutritionally or pharmaceutically active components which do not affect adversely the efficacy of the active ingredients. Some non-limiting embodiments of a synthetic composition of the invention are also described below.

“Therapy” means treatment given or action taken to reduce or eliminate symptoms of a disease or pathological condition.

“Treat” means to address a medical condition or disease with the objective of improving or stabilising an outcome in the person being treated or addressing an underlying nutritional need.

- 5 Treat, therefore, includes the dietary or nutritional management of the medical condition or disease by addressing nutritional needs of the person being treated. “Treating” and “treatment” have grammatically corresponding meanings.

- 10 It has been surprisingly found that human milk oligosaccharides (HMOs) together with inactivated cells of microorganisms synergistically increases the lactate and GABA production in ex vivo model. The result makes plausible that the combination of HMO with inactivated cells of a microorganism or a synthetic composition comprising said combination can be used to promote the production of lactate and/or GABA in the gut of a mammal, preferably a human, when administered enterally.

- 15 Concerning each aspect of the invention, the HMO in the combination may be a single HMO or a mixture of any HMOs. The HMO can be a neutral HMO or an acidic HMO. The neutral HMO is, in one embodiment, one or more non-fucosylated HMOs; in another embodiment the neutral HMO is one or more fucosylated HMOs. Particularly, the fucosylated neutral HMO is selected from the list consisting of 2'-FL, 3-FL, DFL, a LNFP (LNFP-I, LNFP-II, LNFP-III, LNFP-V or LNFP-VI), a LNDFH (LNDFH-I, LNDFH-II or LNDFH-III), FLNH-I, FLNH-II, FLNnH, FpLNH-I and
20 F-pLNnH II, preferably 2'-FL, 3-FL, DFL, a LNFP (preferably LNFP-I) or a LNDFH (preferably LNDFH-I), and the non-fucosylated neutral HMO is selected from the list consisting of LNT, LNnT, LNH, LNnH, pLNH and pLNnH, preferably LNnT or LNT, more preferably LNnT. The acidic (sialylated) HMO is selected from the list consisting of 3'-SL, 6'-SL, FSL, LST a, LST b and LST c, preferably 3'-SL and 6'-SL.

- 25 In one embodiment, the HMO comprises neutral HMOs, preferably at least a first neutral HMO and at least a second neutral HMO, wherein the first neutral HMO is a fucosylated neutral HMO as disclosed above and the second neutral HMO is a non-fucosylated neutral HMO as disclosed above. The fucosylated neutral HMO(s) and the non-fucosylated neutral HMO(s) may be present in a mass ratio of about 4:1 to 1:1. Preferably, the mixture of neutral HMOs contains,
30 consists of or consists essentially of, a fucosylated HMO selected from the list consisting of 2'-FL, 3-FL and DFL, and a non-fucosylated neutral HMO selected from the list consisting of LNT and LNnT; advantageously the mixture comprises, consists of or consists essentially of, 2'-FL and at least one of LNnT and LNT; or at least one of 2'-FL and DFL and at least one of LNnT and LNT; or 2'-FL, DFL and at least one of LNnT and LNT; most preferably 2'-FL and LNnT.

In other embodiment, the neutral HMOs comprises at least a first fucosylated neutral HMO and a second fucosylated neutral HMO. Preferably, the mixture of fucosylated neutral HMOs contains, consists of or consists essentially of, a first fucosylated neutral HMO selected from the list consisting of 2'-FL and 3-FL, and a second fucosylated neutral HMO selected from the list consisting of DFL and LNFP-I; advantageously the mixture comprises, consists of or consists essentially of, 2'-FL and DFL; or 2'-FL and LNFP-I. Further preferably, 2'-FL and DFL may be present in a mass ratio of about 4:1 to 19:1, such as about 9:1, or 2'-FL and LNFP-I may be present in a mass ratio of about 1:1 to 5:1, such as about 2:1.

In a further embodiment, the mixture of HMOs comprises, consists of or consists essentially of, at least a first (acidic) HMO as disclosed above and at least a second (neutral) HMO as disclosed above. Advantageously, the mixture comprises, consists of or consists essentially of, 2'-FL and 3'-SL; or LNnT and 3'-SL; or 2'-FL, LNnT and 3'-SL.

In the context of each aspect of the present invention, including in relation with the preferred HMO embodiments disclosed above, the inactivated cells of a microorganism are inactivated microbial cells that - optionally with cell components and/or metabolites - confer a health benefit on the host when administered, and usually referred to as postbiotic. It is made from the living cells of that microorganism in an inactivation process, preferably by means of thermal inactivation (pasteurization, tyndallization, autoclaving), but alternative inactivation method may also be applied, such as using electric field, ultrasonication, high pressure, X-ray, pulsed light, magnetic field heating or plasma technology. The inactivation method makes the vast majority of the cells lifeless (dead or killed): at least 90 %, preferably at least 95 %, more preferably at least 98 %, even more preferably at least 99 %, advantageously substantially all cell of the microorganism is dead or killed, more advantageously the entire population of the microorganism is dead or killed.

When combined with the HMO, preferably a preparation of the inanimate microorganism is used. Said preparation is typically made by concentration and then optionally, but preferably drying the dead cells together with the fermented culture medium after inactivation to a powder (that is removing water from the medium). As a result, the preparation of the inactivated cells contains substances other than the intact cells, for example cell components (pili, cell wall components, etc.), fermentation additives like carbon and/or energy source used in the fermentation, microbial metabolites and/or end products like amino acids, peptides. The drying method comprises spray-drying, lyophilization, fluid bed drying or other appropriate process. Optionally, in one embodiment, a suitable carrier, for example lactose is added to preparation before or during drying.

Preferably, the living strain of the microorganism the cells of which are to be inactivated is not considered to be a probiotic. With other words, the inactivated cells is not derived from a probiotic strain.

Also preferably, the inactivated cells of the microorganism is derived from a *Lactobacillus* or
5 *Bifidobacterium*, more preferably *Lactobacillus*, for example *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. johnsonii*, *L. fermentum* or *L. reuteri*, more preferably the strains of the above mentioned *Lactobacilli* or *Bifidobacteria* are not considered to be probiotic strains.

In a more preferred embodiment, the *Lactobacillus* is *L. fermentum* (*Limosilactobacillus*
10 *fermentum*) as deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) with the reference code I-2998. In other preferred embodiment, the *Lactobacillus* is *L. delbrueckii* as deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) with the reference code I-4831.

In a particularly preferred embodiment, the *Lactobacillus* comprises or consists of two
15 independent species: *L. fermentum* and *L. delbrueckii*. The ratio of *L. fermentum* to *L. delbrueckii* by weight or cell count may be any suitable ratio from about 99:1 to about 1:99, e.g. about 9:1 to 1:9, including 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, particularly about 9:1-19:1. In an advantageous embodiment, the *Lactobacillus* is *Lactobacillus LB* consisting of *L. fermentum* CNCM I-2998 and *L. delbrueckii* CNCM I-4831, preferably in about a 9:1-19:1 ratio (by intact
20 cell count). The *Lactobacillus LB* may conveniently be isolated from human faeces.

Lactobacillus LB in fermented culture medium (together with components of the culture medium fermentation including peptides, amino acids carbohydrates carbon and minerals) is deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) with the reference code MA 65/4E. Dead *Lactobacillus LB* cells may be obtained by heating the live cells in fermented
25 culture medium at about 110 °C for about 1 hour.

The inactivated cells of a microorganism in the combination concerning all aspects of the present invention, such as in particular the mixture of the inactivated cells of *Limosilactobacillus fermentum* (previously known as *Lactobacillus fermentum*) and the inactivated cells
Lactobacillus delbrueckii together with their culture media can be used in the form of a powder,
30 which can be prepared by drying the dead cells together with the fermented culture medium by conventional drying methods such as by lyophilization, spray-drying, freeze drying or fluid-bed drying prior to formulating into a suitable composition for use according to the present invention. In a particular aspect, lactose may be added to the wet fermented product prior to drying. In another aspect, lactose may also be added in the course of drying as part of the formulation
35 step.

In the combination according to any of the aspects of the invention, the ratio of the inanimate cells in proportion to the weight of the HMO can be any suitable ratio, preferably around 0.5 billion cells per g of HMO or more, more preferably around 1 billion cells per g of HMO or more, such as 1-25 or 1-10 billion cells per g of HMO, more preferably around 4-5 billion cells per g of HMO or more, up to 60-80 billion cells per g of HMO. This relates also to all preferred embodiments of the combination disclosed above.

The invention relates to, also particularly, a combination consisting of a fucosylated HMO, preferably 2'-FL, 3-FL or DFL, more preferably 2'-FL, and dead or inactivated *Lactobacillus LB* cells in or with their dried fermentation medium comprising medium components and optionally lactose, advantageously wherein the combination comprises about 1-10, preferably 4-5 billion cells per g of fucosylated HMO, preferably 2'-FL, 3-FL or DFL, more preferably 2'-FL.

In especially preferred embodiments of the present invention, the inactivated cells of *Limosilactobacillus fermentum* (CNCM I-4831) and *Lactobacillus delbrueckii* (CN-I2998) (together known as *Lactobacillus LB* (CNCM MA 65/4E)) along with their culture media is used in powder form, preferably comprising lactose as carrier material, even more preferably comprising around 60 billion inactivated bacteria per g of powder. Said powder form is e.g. commercially available under the tradename LBiome™ or Humiome® Post LB at dsm-firmenich.

The synthetic composition comprising a combination of an HMO and inactivated cells of a microorganism, including the preferred and more preferred embodiments as disclosed above, can be a pharmaceutical composition. The pharmaceutical composition can contain a pharmaceutically acceptable carrier, e.g. phosphate buffered saline solution, mixtures of ethanol in water, water and emulsions such as an oil/water or water/oil emulsion, as well as various wetting agents or excipients. The pharmaceutical composition can also contain other materials that do not produce an adverse, allergic or otherwise unwanted reaction when administered to humans. The carriers and other materials can include solvents, dispersants, coatings, absorption promoting agents, controlled release agents, and one or more inert excipients, such as starches, polyols, granulating agents, microcrystalline cellulose, diluents, lubricants, binders, and disintegrating agents. If desired, tablet dosages of the anti-infective compositions can be coated by standard aqueous or non-aqueous techniques.

The pharmaceutical compositions can be administered orally, e.g. as a tablet, capsule, or pellet containing a predetermined amount, or as a powder or granules containing a predetermined concentration or a gel, paste, solution, suspension, emulsion, syrup, bolus, electuary, or slurry, in an aqueous or non-aqueous liquid, containing a predetermined concentration. Orally administered compositions can include binders, lubricants, inert diluents, flavouring agents, and

humectants. Orally administered compositions such as tablets can optionally be coated and can be formulated to provide sustained, delayed or controlled release of the mixture therein.

The pharmaceutical compositions can also be administered by rectal suppository, aerosol tube, naso-gastric tube or direct infusion into the GI tract or stomach.

5 The pharmaceutical compositions can also include therapeutic agents such as antiviral agents, antibiotics, probiotics, analgesics, and anti-inflammatory agents. The proper dosage of these compositions for a human can be determined in a conventional manner, based upon factors such immune status, body weight and age. In some cases, the dosage will be at a concentration similar to that found for the HMO in human breast milk. The required amount would generally be
10 in the range from about 200 mg to about 20 g per day, in certain embodiments from about 300 mg to about 15 g per day, from about 400 mg to about 10 g per day, in certain embodiments from about 500 mg to about 10 g per day, in certain embodiments from about 1 g to about 10 g per day. The pharmaceutical composition contains the dead cells so that they amount about 1-60 billion per g of HMO(s) in the composition.

15 The synthetic composition can preferably be a nutritional composition. It can contain sources of protein, lipids and/or digestible carbohydrates and can be in powdered or liquid forms. The composition can be designed to be the sole source of nutrition or a nutritional supplement. The nutritional composition is preferably for non-therapeutic use.

Suitable protein sources include milk proteins, soy protein, rice protein, pea protein and oat
20 protein, or mixtures thereof. Milk proteins can be in the form of milk protein concentrates, milk protein isolates, whey protein or casein, or mixtures of both. The protein can be whole protein or hydrolysed protein, either partially hydrolysed or extensively hydrolysed. Hydrolysed protein offers the advantage of easier digestion which can be important for humans with inflamed GI tracts. The protein can also be provided in the form of free amino acids. The protein can
25 comprise about 5 % to about 30 % of the energy of the nutritional composition, normally about 10 % to 20 %.

The protein source can be a source of glutamine, threonine, cysteine, serine, proline, or a combination of these amino acids. The glutamine source can be a glutamine dipeptide and/or a glutamine enriched protein. Glutamine can be included due to the use of glutamine by
30 enterocytes as an energy source. Threonine, serine and proline are important amino acids for the production of mucin. Mucin coats the GI tract and can improve mucosal healing. Cysteine is a major precursor of glutathione, which is key for the antioxidant defences of the body.

Suitable digestible carbohydrates include maltodextrin, hydrolysed or modified starch or corn starch, corn syrup, corn syrup solids, high fructose corn syrup, rice-derived carbohydrates, pea-
35 derived carbohydrates, potato-derived carbohydrates, tapioca, sucrose, glucose, fructose,

sucrose, lactose, honey, sugar alcohols (e.g., maltitol, erythritol, sorbitol), or mixtures thereof. Generally digestible carbohydrates provide about 35 % to about 55 % of the energy of the nutritional composition. A particularly suitable digestible carbohydrate is a low dextrose equivalent (DE) maltodextrin.

- 5 Suitable lipids include medium chain triglycerides (MCT) and long chain triglycerides (LCT). Preferably the lipid is a mixture of MCTs and LCTs. For example, MCTs can comprise about 30 % to about 70 % by weight of the lipids, more specifically about 50 % to about 60 % by weight. MCTs offer the advantage of easier digestion which can be important for humans with inflamed GI tracts. Generally, the lipids provide about 35 % to about 50 % of the energy of the nutritional
10 composition. The lipids can contain essential fatty acids (omega-3 and omega-6 fatty acids). Preferably these polyunsaturated fatty acids provide less than about 30 % of total energy of the lipid source. Decreasing the levels of these polyunsaturated fatty acids is believed to decrease sensitivity to peroxidation; which can be beneficial for humans having inflammatory conditions.

- Suitable sources of long chain triglycerides are rapeseed oil, sunflower seed oil, palm oil, soy
15 oil, milk fat, corn oil, high oleic oils, and soy lecithin. Fractionated coconut oils are a suitable source of medium chain triglycerides. The lipid profile of the nutritional composition is preferably designed to have a polyunsaturated fatty acid omega-6 (n-6) to omega-3 (n-3) ratio of about 4:1 to about 10:1. For example, the n-6 to n-3 fatty acid ratio can be about 6:1 to about 9:1.

- The nutritional composition preferably also includes vitamins and minerals. If the nutritional
20 composition is intended to be a sole source of nutrition, it preferably includes a complete vitamin and mineral profile. Examples of vitamins include vitamins A, B-complex (such as B1, B2, B6 and B12), C, D, E and K, niacin and acid vitamins such as pantothenic acid, folic acid and biotin. Examples of minerals include calcium, iron, zinc, magnesium, iodine, copper, phosphorus, manganese, potassium, chromium, molybdenum, selenium, nickel, tin, silicon,
25 vanadium and boron.

- The nutritional composition can also include a carotenoid such as lutein, lycopene, zeaxanthin, and beta-carotene. The total amount of carotenoid included can vary from about 0.001 µg/ml to about 10 µg/ml. Lutein can be included in an amount of from about 0.001 µg/ml to about 10 µg/ml, preferably from about 0.044 µg/ml to about 5 g/ml of lutein. Lycopene can be included in
30 an amount from about 0.001 µg/ml to about 10 µg/ml, preferably about 0.0185 mg/ml to about 5 g/ml of lycopene. Beta-carotene can comprise from about 0.001 µg/ml to about 10 mg/ml, for example about 0.034 µg/ml to about 5 µg/ml of beta-carotene.

- The nutritional composition preferably also contains reduced concentrations of sodium; for example, from about 300 mg/l to about 400 mg/l. The remaining electrolytes can be present in
35 concentrations set to meet needs without providing an undue renal solute burden on kidney

function. For example, potassium is preferably present in a range of about 1180 to about 1300 mg/l; and chloride is preferably present in a range of about 680 to about 800 mg/l.

The nutritional composition can also contain various other conventional ingredients such as preservatives, emulsifying agents, thickening agents, buffers, fibres and prebiotics (e.g. fructooligosaccharides, galactooligosaccharides), probiotics (e.g. *B. animalis* subsp. *lactis* BB-12, *B. lactis* HN019, *B. lactis* Bi07, *B. infantis* ATCC 15697, *L. rhamnosus* GG, *L. rhamnosus* HNOOI, *L. acidophilus* LA-5, *L. acidophilus* NCFM, *L. fermentum* CECT5716, *B. longum* BB536, *B. longum* AH1205, *B. longum* AH1206, *B. breve* M-16V, *L. reuteri* ATCC 55730, *L. reuteri* ATCC PTA-6485, *L. reuteri* DSM 17938), antioxidant/anti-inflammatory compounds including tocopherols, carotenoids, ascorbate/vitamin C, ascorbyl palmitate, polyphenols, glutathione, and superoxide dismutase (melon), other bioactive factors (e.g. growth hormones, cytokines, TFG- β), colorants, flavours, and stabilisers, lubricants, and so forth.

The nutritional composition can be in the form of a soluble powder, a liquid concentrate, or a ready-to-use formulation. The composition can be fed to a human via a nasogastric tube or orally. Various flavours, fibres and other additives can also be present.

The nutritional compositions can be prepared by any commonly used manufacturing techniques for preparing nutritional compositions in solid or liquid form. For example, the composition can be prepared by combining various feed solutions. A protein-in-fat feed solution can be prepared by heating and mixing the lipid source and then adding an emulsifier (e.g. lecithin), fat soluble vitamins, and at least a portion of the protein source while heating and stirring. A carbohydrate feed solution is then prepared by adding minerals, trace and ultra-trace minerals, thickening or suspending agents to water while heating and stirring. The resulting solution is held for 10 minutes with continued heat and agitation before adding carbohydrates (e.g. the HMOs and digestible carbohydrate sources) and postbiotics. The resulting feed solutions are then blended together while heating and agitating and the pH adjusted to 6.6-7.0, after which the composition is subjected to high-temperature short-time processing during which the composition is heat treated, emulsified and homogenized, and then allowed to cool. Water soluble vitamins and ascorbic acid are added, the pH is adjusted to the desired range if necessary, flavours are added, and water is added to achieve the desired total solid level. Alternatively, the components may be dry-blended under suitably sterile conditions.

The daily dosage of HMOs for nutritional purposes is around 3-7 g, preferably 3-5 g. The nutritional composition contains the dead cells so that they amount about 1-60 billion per g of HMO(s) in the composition.

For a liquid product, the resulting solution can then be aseptically packed to form an aseptically packaged nutritional composition. In this form, the nutritional composition can be in ready-to-

feed or concentrated liquid form. Alternatively, the composition can be spray-dried and processed and packaged as a reconstitutable powder.

When the nutritional product is a ready-to-feed nutritional liquid, the total concentration of HMOs in the liquid, by weight of the liquid, is from about 0.0001 % to about 2.0 %, including from about 0.001 % to about 1.5 %, including from about 0.01 % to about 1.0 %. When the nutritional product is a concentrated nutritional liquid, the total concentration of HMOs in the liquid, by weight of the liquid, is from about 0.0002 % to about 4.0 %, including from about 0.002 % to about 3.0 %, including from about 0.02 % to about 2.0 %.

The nutritional composition can also be in a unit dosage form such as a capsule, tablet or sachet. For example, the synthetic composition can be in a tablet form comprising the HMOs, and one or more additional components to aid formulation and administration, such as diluents, excipients, antioxidants, lubricants, colorants, binders, disintegrants, and the like.

Suitable diluents, excipients, lubricants, colorants, binders, and disintegrants include polyethylene, polyvinyl chloride, ethyl cellulose, acrylate polymers and their copolymers, hydroxyethyl-cellulose, hydroxypropylmethyl-cellulose (HPMC), sodium carboxymethylcellulose, polyhydroxyethyl methylacrylate (PHEMA), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene oxide (PEO), or polyacrylamide (PA), carrageenan, sodium alginate, polycarbophil, polyacrylic acid, tragacanth, methyl cellulose, pectin, natural gums, xanthan gum, guar gum, karaya gum, hypromellose, magnesium stearate, microcrystalline cellulose, and colloidal silicon dioxide. Suitable antioxidants are vitamin A, carotenoids, vitamin C, vitamin E, selenium, flavonoids, polyphenols, lycopene, lutein, lignan, coenzyme Q10 ("CoQ10") and glutathione.

The unit dosage forms, especially those in sachet form, can also include various nutrients including macronutrients.

Another aspect of the invention relates to the non-therapeutic use of the combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, or the synthetic composition comprising said combination, as defined in the second aspect of the invention, for promoting or enhancing the lactate and/or GABA production in the gut of a mammal, preferably a human, more preferably a non-infant human. Promoting the lactate and/or GABA production in the gut of a mammal who is in a healthy state and/or in a state in which the mammal is not likely to develop any pathological condition contributes to regulating metabolism of the consumer and the stability of the gut microbiome, and thus resulting in improvement of the quality of life and perception of well-being.

Another aspect of the invention relates to a combination of a human milk oligosaccharide (HMO) and inactivated cells of a microorganism, as defined in the first aspect of the invention, or a synthetic composition comprising said combination, as defined in the second aspect of the

invention, for use in a therapy or as a medicament. Preferably, in one embodiment, the use is managing conditions of a mammal wherein enhanced level of lactate production in the gut of the mammal contributes to treat pathological conditions or clinical situations. For example, microbial lactate production in the gut has a role in modulation of host immunity and gut epithelial development or helps the gut microbiota acting as a potent barrier against colonisation and/or proliferation of gastrointestinal pathogens. In other embodiment, the use is managing conditions of a mammal wherein enhanced level of GABA production in the gut of the mammal contributes to treat pathological conditions or clinical situations, preferably one or more of supporting or improving emotional and behavioural development such as reduction of anxiety and/or stress, better mood regulation, improved sleep quality such as better sleep pattern and reduced insomnia, enhanced gut health through regulated improved gut barrier function and/or gut motility, reducing gut inflammation, neuroprotection, immune modulation resulting in better protection against infections and supporting or improving cognitive development, such as motor skills, learning, language skills, and spatial cognition ability. In a particular embodiment, the synthetic composition for use in managing conditions of a mammal wherein enhanced level of GABA production in the gut of the mammal contributes to treat pathological conditions or clinical situations is a nutraceutical composition for use in supporting or improving in a subject one or more of the following:

- cognitive development, such as motor skills, learning, language skills, and spatial cognition ability,
- emotional and behavioural development, such as stress and anxiety reduction,
- a healthy immune system, including immune system development and anti-inflammatory support, and/or
- gut barrier function and/or gut motility.

EXAMPLES

Example 1

A kinetic, *ex vivo* study was implemented, simulating the colonic fermentation of test products by the gut microbiota derived from healthy human infants (exclusively formula-fed, 4-6 months old, n = 12). The exclusion criteria were antibiotic use in 30 days before sample delivery for the study and previous necrotizing enterocolitis (NEC) or gut surgery. Further, there was a preference for infants born via vaginal delivery as opposed to C-section.

Individual bioreactors were processed in parallel in a bioreactor management device (Cryptobiotix, Ghent, Belgium). Each bioreactor contained 5 ml of nutritional medium-faecal inoculum blend dosed with test products, sealed individually, before being rendered anaerobic.

After preparation, bioreactors were incubated under continuous agitation (140 rpm) at 37 °C for 24 hours. Upon gas pressure measurement in the headspace, liquid samples were collected for determination of lactate production with a high-throughput spectrophotometric method.

Test products and doses were:

- 5
- 10
- 2'-FL (assay: 98.2 %; 5 g/l),
 - *Lactobacillus LB* fermentate as lyophilized powder (containing around 55 billion (5.5·10¹⁰) heat-killed, structurally intact *L. fermentum* cells and around 5 billion (5·10⁹) heat-killed, structurally intact *L. delbrueckii* cells per gram together with components of the culture medium fermentation including peptides, amino acids, carbohydrates carbon and minerals, 0.34 g/l), and
 - the mixture of 2'-FL (5 g/l) and *Lactobacillus LB* fermentate as lyophilized powder (0.34 g/l).

The results are given in the table below (mean of 12 tests in each series):

	lactate [mM]
control	0.00
2'-FL	0.98
<i>Lactobacillus LB</i>	0.01
2'-FL + <i>Lactobacillus LB</i>	2.62

- 15
- The results showed that the combination of 2'-FL with dead *Lactobacillus* cells synergistically enhanced the lactate production.

Example 2

- 20
- An *ex vivo* study simulating the colonic fermentation of the test products by the gut microbiota was conducted. Fermentation was done using infant faecal inoculum from 5 voluntary infant donors aged 7-10 months. The fermentations were conducted in a freshly prepared autoclaved minimal colonic media consisting of K₂HPO₄, KH₂PO₄, peptone, mucin, yeast extract, Tween 80 and demineralized H₂O with a 2% faecal inoculum in a sealed 96-well deep-well plate under anaerobic conditions. The plate was left at 37 °C for 24 h before the fermentation was stopped.

- 25
- The samples were analysed to determine GABA content with LC-MS/MS using external standard.

Test products and doses were:

- 2'-FL, 3'-SL, 6'-SL LNnT, LNT LNFP-I, LNDFH-I, 2'-FL:LNnT (4:1 by weight), 2'-FL:DFL (1.8:1 by weight), all in 5 g/l,

- Humiome® Post LB (*Lactobacillus LB* fermentate dried powder with lactose carrier containing around 60 billion ($6 \cdot 10^{10}$) heat-killed cells per gram (combination of structurally intact *L. fermentum* (CNCM I-2998) and *L. delbrueckii* (CNCM I-4831) together with components of the culture medium fermentation including peptides, amino acids, carbohydrates carbon and minerals), 0.34 g/l, and
- the mixture of the HMO (5 g/l) and Humiome® Post LB (0.34 g/l).

The results are given in the table below (mean of 5 tests in each series):

Sample	GABA [μ M]	Sample	GABA [μ M]
control	29	LNT	306
<i>Lactobacillus LB</i>	42	LNT + <i>Lactobacillus LB</i>	675
2'-FL	353	2'-FL:DFL	330
2'-FL + <i>Lactobacillus LB</i>	594	2'-FL:DFL + <i>Lactobacillus LB</i>	633
3'-SL	54	LNFP-I	333
3'-SL + <i>Lactobacillus LB</i>	517	LNFP-I + <i>Lactobacillus LB</i>	550
6'-SL	31	LNDFH-I	210
6'-SL + <i>Lactobacillus LB</i>	454	LNDFH-I + <i>Lactobacillus LB</i>	425
LNnT	199	2'-FL:LNnT	310
LNnT + <i>Lactobacillus LB</i>	429	2'-FL:LNnT + <i>Lactobacillus LB</i>	666

The results showed that the combination of 2'-FL with dead *Lactobacillus* cells synergistically enhanced the GABA production.

CLAIMS

1. A combination comprising, preferably consisting essentially of or consisting of
 - a human milk oligosaccharide (HMO) and
 - inactivated cells of a microorganism, preferably a postbiotic,
- 5 optionally with or in a concentrated or dried fermentation medium from which the microorganism is derived and the medium components therein.
2. The combination according to claim 1, wherein the microorganism is not a probiotic.
3. The combination according to claim 1 or 2, wherein the inactivated cells are comprised in a dried fermentation medium optionally with lactose.
- 10 4. The combination according to any of the precedent claims, wherein the microorganism is *Lactobacillus*, preferably a mixture of *L. fermentum* and *L. delbrueckii*.
5. The combination according to claim 4, wherein the *Lactobacillus* is *Lactobacillus LB* as deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) with the reference code MA 65/4E.
- 15 6. The combination according to any of the precedent claims, wherein the HMO is a neutral HMO, preferably wherein the neutral HMO is fucosylated HMO, a non-fucosylated HMO or a mixture thereof.
7. The combination according to claim 6, wherein the fucosylated HMO is 2'-FL, 3-FL, DFL, a LNFP or a LNDFH, preferably 2'-FL.
- 20 8. The combination according to claim 6, wherein the non-fucosylated HMO is LNT, LNTnT, LNH, LNTnH, pLNH or pLNTnH, preferably LNT or LNTnT.
9. The combination according to any of the claim 1 to 5, wherein the HMO is a sialylated HMO, preferably 3'-SL, 6'-SL, FSL, LST a, LST b or LST c, more preferably 3'-SL and 6'-SL.
- 25 10. The combination according to any of the precedent claims, wherein the ratio of the inactivated cells in proportion to the weight of the HMO is around 1 billion cells per g of HMO or more, preferably around 4-5 billion cells per g of HMO or more, up to 60-80 billion cells per g of HMO.
11. A synthetic composition comprising the combination as defined in any of the precedent
- 30 claims.
12. The combination or the synthetic composition according to any of the precedent claims for use in therapy or as a medicament, preferably for use in treating conditions of a

mammal wherein enhanced level of lactate and/or GABA production in the gut of the mammal contributes to treat pathological conditions or clinical situations.

13. The combination for the use or the synthetic composition for the use according to claim 12, wherein the use is supporting or improving one or more of the following:

- 5 - cognitive development, such as motor skills, learning, language skills, and spatial cognition ability,
- emotional and behavioural development, such as stress and anxiety reduction,
- a healthy immune system, including immune system development and anti-inflammatory support, and/or
- 10 - gut barrier function and/or gut motility.

14. Non-therapeutic use of the combination or the synthetic composition according to any of the precedent claims for promoting or enhancing the lactate and/or GABA production in the gut of a mammal, preferably a human, more preferably a non-infant human.

15 15. The non-therapeutic use according to claim 14, wherein the mammal is in a healthy state and/or in a state in which the mammal is not likely to develop any pathological condition.