# COMBINATION OF MIXTURES OF HMOS WITH MFGM AND THEIR USE

## *FIELD*

The present disclosure relates compositions comprising milk fat globule membrane (MFGM) and a mixture of HMOs with at least one fucosylated HMO and at least one HMO selected from neutral core HMOs or sialylated HMOs. The compositions have potential health benefits in terms of improving brain health, immunity and recovery time following exercise, by increasing the formation of glutamic acid.

## *BACKGROUND*

The human gut microbiome is composed of bacteria, archaea, viruses, and eukaryotic microbes that reside inter alia in the gut. These microbes have tremendous potential to impact our physiology, both in health and in disease. The microbiota of the human intestine is a complex and very dynamic microbial ecosystem, which is considered to serve numerous important functions for its human host, including protection against pathogens, induction of immune regulatory functions, nutrient processing and metabolic functions, these basic functions, affect directly or indirectly most of our physiologic functions. Various gut bacteria, including certain Bifidobacterium spp., can feed on oligosaccharides such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and human milk oligosaccharides (HMOs), whereas these structures are indigestible by humans. Due to the ability to feed beneficial microorganisms and induce the growth or activity of these, certain oligosaccharides are referred to as prebiotics.

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 are present in concentrations of 5-25 g/l. Certain HMOs are believed to be important for the development of the infant gut microbiota in particular by increasing the predominance of bifidobacteria. HMOs have also been shown to have beneficial effects in maintaining the gut microbiota in adults where they are known as prebiotics and are known to increase beneficial *Bifidobacterium* in the gut and support establishment and maintenance of a balanced gut microbiota.

Human breastmilk also contains lipids. Milk fat globule (MFG) are secreted by the mammary gland, the core of the globule consists mainly of triglycerides (98%), which is surrounded by a phospholipid trilayer referred to as a milk fat globule membrane (MFGM). MFGM consists of phospholipids such as sphingomyelins, phosphatidylcholines, gangliosides and different proteins, including lactoferrin or mucins. Evidence indicates that MFGM can exert a beneficial effect neurodevelopment and defense against infections (See for example Hernell et al 2016 J Pediatr 173 Suppl:S60-5 doi: 10.1016/j.jpeds.2016.02.077). MFGM also have benefits in adults, such as stimulation of neuromuscular functions and reduced stress and anxiety.

US 11,849,747 describes a combination of MFGM and 2'FL and at least one additional component chosen from the group consisting of DHA, ARA, Vitamin E, Vitamin C, and sphingomyelin.

CN115887626 and CN116391759 describes a composition that contains MFGM, 2'FL and 6'SL or just 6'SL as well as lactoferrin and osteopontin to reduce bacterial infections such as *Staphylococcus aureus and E.coli*.

WO2011/069987 describes nutritional composition with MFGM, probiotics and prebiotics such as FOS and GOS for use in prevention and/or treatment of infections and/or inflammatory conditions, and/or physio-pathological gut discomfort.

WO2021/021746 describes the use of MFGM to deliver probiotics, potentially in combination with prebiotics such as GOS or a single HMO for use in treating intestinal diseases.

***SUMMARY***

The present disclosure relates to a composition comprising milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein said mixture of HMOs comprises at least one fucosylated HMO, and at least one HMO selected from of a neutral core HMO and/or a sialylated HMO, wherein, if 6'-sialyllactose (6'-SL) is present in the mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1.

In embodiments, the fucosylated oligosaccharide is selected from the group consisting of 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), difucosyllactose (DFL), lacto-N-fucopentaose I (LNFP-I), lacto-N-difucohexaose I (LNDFH-I), or a mixture thereof. Preferably, the fucosylated oligosaccharide is a fucosyllactose, such as 2'FL, 3FL or DFL, or a mixture thereof.

The sialylated HMO is preferably sialyllactose, such as 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), or combinations thereof. The neutral core HMO is preferably lacto-N-tetraose (LNT) or lacto-N-neotetraose (LNnT or combinations thereof.

Preferred compositions disclosed herein are MFGM combined with one of the following HMO mixtures:

a. 2'FL and LNT, or
b. 2'FL and LNnT, or
c. 2'FL, LNT, 6'SL, or
d. 2'FL, LNnT and 6'SL, or
e. 2'FL, LNnT 6'SL and 3'SL, or
f. 2'FL, DFL, LNnT, 6'SL and 3'SL, or
g. 2'FL, DFL, LNT, 6'SL and 3'SL, or
h. 2'FL, 3FL, LNT, 6'SL and 3'SL, or
i. 2'FL, DFL, 3'SL and 6'SL.

In further aspects of the disclosure, the composition as described herein is used for supporting or improving brain health, such as improving cognition and/or immunity such as reduced inflammation, including reduced symptoms of irritable, bowel syndrome (IBS) and inflammatory bowel disease (IBD), and/or recovery time following exercise, including reducing muscle fatigue, in a subject.

Furthermore, a composition as described herein may be used to increase the abundance of the glutamic acid/glutamate in the gut of the subject, when compared to a non-administered subject, or the same subject before administration of the composition.

In some embodiments, the subject may be an infant or a non-infant such as a child, or older adult or elderly individual.

***BRIEF DESCRIPTION OF THE DRAWINGS***

**Figure 1** Illustration of milk fat globule membrane (MFGM) structure and components.

**Figure 2** Box plot showing the level of the metabolite L-glutamic acid produced across the different donors with the test compositions of table 1. The No_sugar sample is a control sample with fecal slurry and no test composition. The baseline is indicated as the dotted line and reflect the glutamic acid levels in a medium only sample. The Y axis refers to the relative change in glutamate compared to medium only sample set to 0.

***DETAILED DESCRIPTION***

The present disclosure relates to a composition comprising milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein said mixture of HMOs comprises at least one fucosylated HMO, and at least one HMO selected from of a neutral core HMO and/or a sialylated HMO.

In the present disclosure the effect of the compositions described herein was tested in fecal samples which were subjected to anaerobic fermentation with the compositions of the present disclosure and relevant control compositions. The fecal fermentation allows the microbiota in the samples to interact with the test compositions which is indicative of what would happen in the colon of a subject. The compositions described herein have been found to increase metabolites with associated health benefits.

In particular glutamic acid or glutamate was increased in the fermented fecal samples as shown in example 1. Glutamic acid is a non-essential amino acid which can be produced by the body itself. Glutamic acid has been shown to have various health benefits in areas such as brain health, immunity and recovery after exercise.

Glutamic acid is the main excitatory neurotransmitter in the brain and is involved in many important brain functions such as cognitive functions including learning and memory. In elderly low prefrontal glutamate levels have been connected with impairments in working memory (see

for example Rmuset al eLife 2023;12:e85243. DOI: https://doi.org/10.7554/eLife.85243). In infants and young children glutamic acid plays a critical role in promoting neuronal activity and communication between brain cells. This activity is important for many aspects of brain development, including learning, memory, and the formation of new neural connections. Glutamic acid is therefore beneficial in brain health. In particular in developing infants and children.

As reviewed by Jansson et al 2014, Journal of neural transmission, Volume 121, pages 819–836, glutamate also has a role in the proliferation, migration, differentiation and survival of neural progenitor cells – and is therefore heavily involved in brain development, which is of particular importance for infants.

Glutamic acid is used by immune cells for energy and to support cell growth and division. It may also help to reduce inflammation and improve gut health. Glutamic acid is important for maintaining the health of the intestinal lining and may help to reduce symptoms of conditions such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). In healthy individuals, the concentration of glutamate in the gut is generally in the range of 0.1-1 mM

Glutamic acid may help to improve exercise performance by reducing muscle fatigue and improving recovery time after exercise. Glutamic acid can be converted into glutamine which is known to aid in muscle recovery after exercise (see for example Córdova-Martínez et al 2021 Nutrients 13(6): 2073).

The term "Microbiota", "microflora" and "microbiome" are used interchangeably and refer to a community of living microorganisms that typically inhabits a bodily organ or part in an animal or human. Particularly, in the gastrointestinal organs of animals or humans the microflora is termed the gastrointestinal or gut microbiome or microbiota. The most dominant members of the gastrointestinal microbiota in non-infant humans include 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 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 microbiota, which is found in the lumen of the gastrointestinal tract.

The term “intestine” or “gut” are used interchangeably herein and refers to the portion of the gastrointestinal tract consisting of the small intestine and the large intestine. The “large intestine” (*intestinum crassum*) is the lower part of the gastrointestinal tract and is also referred to herein as “colon”.

The term "Milk fat globule membrane" ("MFGM") as used herein includes lipid components such phospholipids, cerebrosides, gangliosides, sphingoids or sphingolipids, and/or cholesterol. Additionally, "milk fat globule membrane" as used herein may include milk fat globule membrane proteins such as mucin 1 (MUC1), mucin 15 (MUC15)butyrophilin, adipophilin, CD36, CD14, lactadherin (PAS6/7), xanthine oxidase and fatty acid binding proteins. The MFGM proteins have been shown to have health benefits. Mucin1 has protective effects on epithelial cells and may contribute to the prevention of infections and the maintenance of gut health. Butyrophilin is involved in immune regulation and has potential anti-inflammatory properties. Adipophilin is involved in lipid metabolism and storage and it may play a role in metabolic health and the management of obesity-related conditions. CD36 is involved in lipid metabolism and may have implications for cardiovascular health. Lactadherin has been shown to have antiviral properties, particularly against rotavirus, which causes severe diarrhea in infants. Xanthine oxidase has antioxidant properties and can help reduce oxidative stress and may have implications for cardiovascular health. Figure 1 illustrates a non-limiting structure of a milk fat globule membrane. Phospholipids make up 30% of the total lipid weight of the MFGM. Phospholipids include for example phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin. Sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine make up 30% each of the total phospholipid content in MFGM. In embodiments, the MFGM used herein comprise or consist of the lipids found in the natural MFGM membrane including the protein fraction, in particular proteins such as mucin 1, mucin 15, butyrophilin, adipophilin, CD36, lactadherin and xanthine oxidase. In embodiments, the MFGM used herein comprise or consist of the lipids found in the natural MFGM membrane without the protein fraction. In embodiments the phospholipids constitute 30-60% of the MFGM lipid composition, cholesterol constitute 10-30% and glycolipids constitute less than 5%, such as between 0.5 and 3%, other lipids such as sphingolipids and free fatty acids may also be present. In one embodiment the MFGM comprises at least the following lipids: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. MFGM may comprise, consist of, or essentially consist of one or more of each of the following components per 100g dry weight, (a) to (g) per 100g dry weight: (a) Phospholipids: 400-700 mg, wherein the phospholipids may be distributed as in (b) to (f): (b) Phosphatidylcholine: 80-200 mg, (c) Phosphatidylethanolamine: 80-200 mg, (d) Phosphatidylinositol: 20-65 mg, (e) Phosphatidylserine; 30-95 mg, (f) Sphingomyelin: 80-200 mg, (g) Gangliosides: 18-65 mg. It is understood that the MFGM used in the present disclosure

is different from what is found in mothers milk, since the structure changes through the isolation process. In one embodiment the MFGM is isolated bovine, buffalo, horse, goat and/or human milk. In embodiments MFGM may be supplied by the inclusion of an enriched milk product, such as an enriched whey protein concentrate (eWPC) in the nutritional composition. Enriched milk product generally refers to a milk product that has been enriched with certain milk fat globule membrane (MFGM) components, such as proteins and lipids found in the MFGM. The enriched milk product can be formed by, e.g., fractionation of non-human (e.g., bovine) milk. Enriched milk products have a total protein level which can range between 20% and 90%, more preferably between 68% and 80%, of which between 3% and 50% is MFGM proteins; MFGM proteins may make up from 7% to 13% of the enriched milk product protein content. Enriched milk products also comprise from 0.5% to 5% (and, at times, 1.2% to 2.8%) sialic acid, from 2% to 25% (and, in some aspects, 4% to 10%) phospholipids, from 0.4% to 3% sphingomyelin, from 0.05% to 1.8%, and, in certain aspects 0.10% to 0.3%, gangliosides and from 0.02% to about 1.2%, more preferably from 0.2% to 0.9%, cholesterol. MFGM is also available from commercial sources such as Lacprodan® MFGM-10 and Lacprodan PL-20 from Arla, NutriPRO™ Milk Fat Globule Membrane (MFGM) from Milk Specialities Global (MSG), MFGM from Univar Solutions, SureStart™ MFGM Lipid 70/100 from NZMP Fonterra Co-operative and Vivinal® MFGM from Friesland Campina. These products may also contain some protein and carbohydrates which are not necessarily incorporated into the MFGM structure but just remnants from the milk they are produced from. Components that are not incorporated into the MFGM membrane structure as depicted in figure 1 are not considered to be part of the MFGM.

In the context of the present disclosure, the term “probiotic” refers to microbial cells or cell preparations, such as bacteria, which, when ingested in adequate amounts, provide a benefit to the host (human or animal) by replenishing or otherwise supplementing the natural gastrointestinal flora or by eliminating undesired bacteria in the gastrointestinal (GI) tract or by executing beneficial metabolic activities along the GI tract or by stimulating the immune system. In some embodiments the compositions disclosed herein do not comprise a probiotic, in that the composition naturally increases beneficial bacteria in the gut microbiome, such as *Bifidobacterium.*

In the context of the present disclosure, bacteria belonging to the *Bifidobacterium* genus may be selected from, without being limited to, the following *Bifidobacterium* sp.: *B. adolescentis*, *B. angulatum*, *B. animalis*, *B. animalis subsp. animalis*, *B. animalis subsp. lactis*, *B. asteroides*, *B. biavatii*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. coagulans*, *B. longum*, *B. infantis*, *B. longum subsp. infantis*, *B. longum subsp. longum*, *B. magnum*, *B. coryneforme*, *B. dentium*, *B. gallicum*, or *B. subtile*. In a preferred embodiment, the *Bifidobacterium* sp is selected from *B. bifidum* or *B. longum* such as *B. longum subsp. Infantis.*

In relation to any of the uses described herein, which result in a benefit, such as an increase, it is understood that this is compared to the same subject prior to the administration of the composition described herein. Alternatively, the comparative parameters may also constitute two cohorts of individuals one receiving a composition described herein and one or more additional cohorts receiving a placebo, such as a maltodextrin or lactose or alternative the same mixture of HMOs without MFGM. The term cohort in this respect is understood as groupings of individuals with common traits, such as age, social and health factors. The size of a cohorts needed for comparative studies depends on the statistical variation observed within a cohort.

"Prebiotic" is a term used to describe compounds in food that induce the growth or activity of beneficial microorganisms such as bacteria and fungi in the gut of an animal species or a human. In the gastrointestinal tract, prebiotics can alter the composition of organisms in the gut microbiome. Dietary prebiotics are typically nondigestible fiber compounds that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth or activity of advantageous bacteria in the colon by acting as substrates for them. Common prebiotics used in food manufacturing include beta-glucan from oats and inulin from chicory root. Oligosaccharides that are undigestible by humans and animals, like fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) and polydextrose (PDX) and human milk oligosaccharides (HMOs). These may act as prebiotics for certain bacterial species in the gut. In the context of the present disclosure the composition preferably only contains a mixture of HMOs as prebiotic. Preferably, the composition does not contain GOS or PDX.

In the context of the disclosure, the term "oligosaccharide" means a saccharide polymer containing a number of monosaccharide units. In some embodiments, preferred oligosaccharides are saccharide polymers consisting of three to nine monosaccharide units, preferred oligosaccharides are tri-saccharides, tetra-saccharides, penta-saccharides or hexa-saccharides. In relation to the present disclosure the oligosaccharides are fucosylated oligosaccharides, preferably a neutral fucosylated human milk oligosaccharide.

The term "human milk oligosaccharide" or "HMO" in the present context refers to a complex carbohydrate found in human breast milk. The HMOs have a core structure comprising a lactose unit at the reducing end that can be elongated by one or more beta-N-acetyl-lactosaminyl and/or one or more beta-lacto-N-biosyl unit, and this core structure can be substituted by an alpha-L-fucopyranosyl and/or an alpha-N-acetyl-neuraminyl (sialyl) moiety. HMO structures are e.g., disclosed by Xi Chen in Chapter 4 of Advances in Carbohydrate Chemistry and Biochemistry 2015 vol 72.

In the context of the present disclosure the disaccharides lactose, N-acetyllactosamine or lacto-N-biose are not regarded as an HMO species.

HMOs can be non-acidic (or neutral) or acidic. Neutral HMOs are devoid of a sialyl residue and acidic have at least one sialyl residue in their structure. The non-acidic (or neutral) HMOs can be fucosylated or non-fucosylated.

Examples of such neutral non-fucosylated (neutral core) HMOs include lacto-N-triose II (LNT-II) 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), 3-fucosyllactose (3FL), difucosyllactose (DFL), lacto-N-fucopentaose I (LNFP-I), lacto-N-difucohexaose I (LNDFH-I), 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-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-neohexaose (FLNnH).

Examples of acidic HMOs or sialylated HMOs include 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), 3-fucosyl-3'-sialyllactose (FSL), 3'-O-sialyllacto-N-tetraose a (LST a), fucosyl-LST a (FLST a), 6'-O-sialyllacto-N-tetraose b (LST b), fucosyl-LST b (FLST b), 6'-O-sialyllacto-N-neotetraose (LST c), fucosyl-LST c (FLST c), 3'-O-sialyllacto-N-neotetraose (LST d), fucosyl-LST d (FLST d), 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).

The production of various fucosylated oligosaccharides and certain particular HMOs is well known. See for example Bych et al 2019 Current Opinion in Biotechnology 56:130–137 for a review on HMO production. In a preferred embodiment, a fucosylated oligosaccharide, such as the fucosylated HMO of the present disclosure may be produced synthetically meaning it is produced *ex vivo* chemically and/or biologically, e.g., by means of chemical reaction, enzymatic reaction or from recombinant cell cultures. For example, various fucosylated oligosaccharides can be made as described in WO2012/127410, WO 2010/115934, WO 2010/115935, WO 2013/139344 or PCT/EP2021/086932.

The composition described herein comprise a mixture of human milk oligosaccharides (HMOs) and milk fat globule membrane (MFGM). The mixture of HMOs in the compositions described herein comprises a) at least one fucosylated HMO, and b) at least one additional HMO selected from of a neutral core HMO and/or a sialylated HMO. Preferably, if 6'-sialyllactose (6'-SL) is present in the HMO mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1, such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1. Even more preferably at least one fucosylated HMO is 2'FL and if 6'-sialyllactose (6'-SL) is present in the mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1 , such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1.

In an alternative embodiment the mixture of HMOs in the compositions described herein comprises: a) at least one fucosylated HMO, and b) at least one additional HMO selected from of a neutral core HMO and/or a sialylated HMO, with the proviso that if the mixture contains 2'FL and a neutral core HMO is not present any 6'-SL present in the HMO mixture would be in a ratio (w/w) of 2'FL:6'-SL that is above 4:1, such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1.

In one or more preferred embodiments, the at least one fucosylated HMO is selected from the group consisting of 2'FL, 3FL, DFL, FSL, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II and LNDFH-III or a mixture thereof.

In selected embodiment the fucosylated HMO is selected from the group of 2'FL, 3FL, DFL, LNFP-I and LNDFH-I, or a mixture thereof. More preferably the fucosylated HMO is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof. Most preferably the fucosylated HMO comprises or consists of 2'FL and DFL.

In embodiments the HOM mixture comprises or consists of a fucosylated HMO and at least one neutral core HMO. The neutral core HMO is preferably selected from LNT-II, LNT and/or LNnT, more preferably the neutral core HMO is LNT and/or LNnT.

In one embodiment the mixture of HMOs comprises or consists of 2'FL and DFL at least one additional HMO selected from of a neutral core HMO.

In embodiments the HMO mixture comprises or consists of a fucosylated HMO and at least one sialylated HMO. The sialylated HMO is preferably selected from 3'SL, 6'SL, LST a, LST b, LST c and FSL, or a mixture thereof. More preferably the sialylated HMO is selected from 3'SL or 3'SL and 6'SL.

In one embodiment the mixture of HMOs comprises or consists of 2'FL and DFL at least one additional HMO selected from 3'SL or 3'SL and 6'SL or 6'-SL in the ratio (w/w) of the 2'FL:6'-SL is above 4:1 , such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and LNT.

In another embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and LNnT.

In another embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and LNT and LNnT.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with LNnT.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with LNT.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with LNT and LNnT.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and 3'SL.

In another embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and 6'SL.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof and 3'SL and 6'SL.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with 3'SL.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with 6'SL.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with 3'SL and 6'SL.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof combined with 6'SL and LNT.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof combined with 6'SL and LNnT.

In one embodiment the mixture of HMOs is 2'FL combined with 6'SL and LNT or LNnT.

In one embodiment the mixture of HMOs is a fucosyllactose, such as 2'FL, 3FL, DFL or a mixture thereof combined with 3'SL and 6'SL and LNT or LNnT.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with 3'SL and 6'SL and LNnT.

In another embodiment the mixture of HMOs is 2'FL alone or 2'FL and DFL combined with 3'SL and 6'SL and LNT.

In one embodiment the mixture of HMOs is 2'FL and 3FL combined with 3'SL and 6'SL and LNT or LNnT.

In one embodiment the mixture of HMOs is 2'FL and 3FL combined with 3'SL and 6'SL and LNT.

In one embodiment the mixture of HMOs is 2'FL and 3FL combined with 3'SL and 6'SL and LNnT.

In preferred embodiments the fucosylated HMO(s) constitute at least 35 wt%, such as at least 40 wt% such as at least 45 wt%, such as at least 50 wt% of the mixture of HMOs. Preferably, 2'FL constitute at least 35 wt%, such as at least 40 wt%, such as at least 45 wt%, such as at least 50 wt%, of the mixture of HMOs.

In some embodiments, the mixture of HMOs constitutes at least at least 90 wt%, such as at least 95 wt%, such as at least 98 wt% of the total amount of HMOs in the composition, and the HMO mixture is selected from a mixture comprising or consisting of

a) 2'-FL and 3'-SL, in a ratio between 9.5-18:1 of 2'-FL:3'-SL, such as between 11-16:1, or
b) 2'-FL and 6'-SL, in a ratio between 4-10:1 of 2'-FL:6'-SL, such as between 4.5-8:1, such as between 5.5-7:1, or
c) 2'-FL and LNnT, in a ratio between 2-8:1 of 2'-FL:LNnT, such as between 4-6.5:1, or
d) 2'-FL and LNT, in a ratio between 1.5-6:1 of 2'-FL:LNT, such as between 2-4:1, or
e) 2'-FL, LNT and LNnT, in a ratio between 4-8:1-3:1 of 2'-FL:LNT:LNnT, such as between 5-7:1.5-2.5:1, or
f) 2'-FL, LNnT and DFL, in a ratio between 6-11:0.5-3:1 of 2'-FL:LNnT:DFL, such as between 7-9:1-2:1, or
g) 2'-FL, LNT and DFL, in a ratio between 6-11:0.5-4:1 of 2'-FL:LNT:DFL, such as between 7-9:1.5-3:1, or
h) 2'-FL, LNnT and 3'-SL, in a ratio between 10-18:1-5:1 of 2'-FL:LNnT:3'-SL, such as between 12-16:2-4:1 or
i) 2'-FL, 6'-SL and LNnT, in a ratio between 3-9:0.5-2:1 of 2'-FL:6'-SL:LNnT, such as between 4-8:1-1.5:1 or
j) 2'-FL, LNT and 3'-SL, in a ratio between 8-16:2-6:1 of 2'-FL:LNT:3'-SL, such as between 10-14:3-5:1 or
k) 2'-FL, LNT and 6'-SL, in a ratio between 4-8:1-4:1 of 2'-FL:LNT:6'-SL, such as between 5-7:1-3:1, or
l) 2'-FL, DFL and 3'-SL, in a ratio between 6-10:0.5-3:1 of 2'-FL:DFL:3'-SL, such as between 7-9:1-2:1, or
m) 2'-FL, DFL and 6'-SL, in a ratio between 5-10:0.5-3:1 of 2'-FL:DFL:6'-SL, such as between 7-9:1-2:1, or
n) 2'-FL, DFL, 6'-SL and 3'SL, in a ratio between 8-16:0.5-3:1-4:1 of 2'-FL:DFL:6'-SL:3'SL, such as between 9-14:1-2:1.5-3:1, or
o) 2'-FL, LNnT, 6'-SL and 3'SL, in a ratio between 8-16:2-6:1-4:1 of 2'-FL:LNnT:6'-SL:3'SL, such as between 9:14:3-5:1-3:1, or
p) 2'-FL, LNT, 6'-SL and 3'SL, in a ratio between 8-16:1-4:1-4:1 of 2'-FL:LNT:6'-SL:3'SL, such as between 9:14:1-3:1-3:1, or

q) 2'-FL, 3FL, LNT, 6'-SL and 3'-SL, in a ratio between 8-16:2-6:2-6:0.5-4:1 of 2'-FL:3FL:LNT:6'-SL:3'-SL, such as between 10-14:3-5:3-5:1-3:1

r) 2'-FL, DFL, LNnT, 6'-SL and 3'-SL, in a ratio between 8-16:1-3:0.5-4: 0.5-4:1 of 2'-FL:DFL:LNnT:6'-SL:3'-SL, such as between 10-14:1-2:3-5:3-5:1.

In embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein the mixture of HMOs is selected from one of the following mixtures:

a. 2'FL and LNT, or
b. 2'FL and LNnT, or
c. 2'FL, LNT, 6'SL, or
d. 2'FL, LNnT and 6'SL, or
e. 2'FL, LNnT 6'SL and 3'SL, or
f. 2'FL, DFL, LNnT, 6'SL and 3'SL, or
g. 2'FL, DFL, LNT, 6'SL and 3'SL, or
h. 2'FL, 3FL, LNT, 6'SL and 3'SL, or
i. 2'FL, DFL, 3'SL and 6'SL, and

wherein the selected mixture of HMOs constitutes at least 90 wt%, such as at least 95 wt%, such as at least 98 wt% of the total amount of HMOs in the composition.

In preferred embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs) comprising 2'FL, DFL, 3'SL and 6'SL, wherein the mixture of HMOs constitutes at least 90 wt%, such as at least 95 wt%, such as at least 98 wt% of the total amount of HMOs in the composition.

In embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein the mixture of HMOs consists essentially of:

a. 2'FL and LNT, or
b. 2'FL and LNnT, or
c. 2'FL, LNT, 6'SL, or
d. 2'FL, LNnT and 6'SL, or
e. 2'FL, LNnT 6'SL and 3'SL, or
f. 2'FL, DFL, LNnT, 6'SL and 3'SL, or
g. 2'FL, DFL, LNT, 6'SL and 3'SL, or
h. 2'FL, 3FL, LNT, 6'SL and 3'SL, or
i. 2'FL, DFL, 3'SL and 6'SL.

In preferred embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), consisting essentially of 2'FL, DFL, 3'SL and 6'SL.

In embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein the mixture of HMOs comprises or consists essentially of:

a. 60 wt% to 80 wt% 2'FL and 20 wt% to 40 wt% LNT, or
b. 65 wt% to 90 wt% 2'FL and 10 wt% to 35 wt% LNnT, or
c. 60 wt% to 75 wt% 2'FL, 15 wt% to 25 wt% LNT, 5 wt% to 15 wt% 6'SL, or
d. 65 wt% to 85 wt% 2'FL, 5 wt% to 20 wt% LNnT and 8 wt% to 20 wt% 6'SL, or
e. 60 wt% to 75 wt% 2'FL, LNnT 6'SL and 3'SL, or
f. 60 wt% to 75 wt% 2'FL, 5 wt% to 12 wt% DFL, 5 wt% to 15 wt% LNnT, 5 wt% to 15 wt% 6'SL and 2 wt% to 10 wt% 3'SL, or
g. 50 wt% to 70 wt% 2'FL, 3 wt% to 12 wt% DFL, 12 wt% to 25 wt% LNT, 5 wt% to 15 wt% 6'SL and 2 wt% to 10 wt% 3'SL, or
h. 45 wt% to 65 wt% 2'FL, 10 wt% to 20 wt% 3FL, 10 wt% to 25 wt% LNT, 5 wt% to 15 wt% 6'SL and 2 wt% to 8 wt% 3'SL, or
i. 45 wt% to 70 wt% 2'FL, 2 wt% to 10 wt% DFL, 5 wt% to 15 wt% 6'SL and 5 wt% to 15wt% 3'SL, and

wherein the selected mixture of HMOs constitutes at least 95 wt%, such as at least 97% wt%, such as at least 98 wt%, such as at least 99 wt%, such as 100 wt% of the total amount of HMOs in the composition.

In preferred embodiments of the present disclosure the composition comprises milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein the mixture of HMOs comprises or consists essentially of 45 wt% to 70 wt% 2'FL, 2 wt% to 10 wt% DFL, 5 wt% to 15 wt% 6'SL and 5 wt% to 15wt% 3'SL, and wherein the selected mixture of HMOs constitutes at least 95 wt%, such as at least 97% wt%, such as at least 98 wt%, such as at least 99 wt%, such as 100 wt% of the total amount of HMOs in the composition.

The composition of the present disclosure may comprise or contain about 25 to 50 wt% of MFGM and between 50 to 75 wt% of the HMO mixture.

In one embodiment the MFGM is primarily composed of lipids, such as between 70 to 100% lipids. In a further embodiment the phospholipids constitute 20-50% of the MFGM and cholesterol constitute 3-10%. Furthermore, glycolipids may constitute between 0.5 and 5%, such as between 1% and 3% of the MFGM, other lipids such as sphingolipids and fatty acids may constitute the reminder of the lipids in the MFGM. In embodiments the MFGM comprises or consists of the phospholipids, phosphatidylcholine, phosphatidylethanolamine,

phosphatidylserine and sphingomyelin. Preferably, the MFGM phospholipids in the comprises 20-30% phosphatidylcholine and 20-30% phosphatidyl-ethanolamine and 5-15% phosphatidylserine and 15-30% sphingomyelin.

The composition of the present disclosure may comprise additional ingredients, such as nutritional ingredients, fillers or carriers. In such compositions the ratio between MFGM and the HMO mixture is between 1 and 3, such as between 1 and 2.5, such as between 1 and 2, such as between 1 and 1.5, such as 1:1.

In preferred embodiments the compositions of the present disclosure may constitute non-HMO oligosaccharides (fiber), such as FOS, GOS and/or PDX. Preferably, non-HMO oligosaccharides constitute less than 10 wt%, such as less that 5 wt%, such as less that 2 wt% of the composition of the present disclosure. Preferably the composition of the present disclosure is essentially free of FOS, GOS and PDX.

In some embodiments the MFGM component and the mixture of HMOs is formulated together. Alternatively, the MFGM component and the mixture of HMOs may be formulated as two separate compounds (e.g., a kit of parts) which are then mixed in the final product, such as a nutritional or pharmaceutical product.

The composition of the present disclosure may be in the form of a pharmaceutical composition or medical food.

The composition of the present disclosure is preferably a nutritional composition such as a dietary supplement or an infant formula or growing up milk or food supplement. It is understood that a nutritional composition refers to a formulated mixture of individual ingredients designed to provide essential dietary elements. A synthetic nutritional composition is a composition where at least one of the ingredients is obtained by a biological process (e.g., enzymatic or fermentation) or chemical process. In the context of the present disclosure mothers milk is not considered to be a nutritional composition since it is not formulated from individual ingredients. In preferred embodiments the nutritional composition is a synthetic nutritional composition.

In a nutritional composition as disclosed herein, the MFGM is preferably added in an amount of 0.2 to 10 mg/ml, such as 0.5 to 5 mg/ml, such as 1 to 10 mg/ml, such as 1.5 mg/ml to about 7.5 mg/ml and the HMO mixture is preferably added in an amount of 0.2 to 20 mg/ml, such as 0.5 to 10 mg/ml, such as 1 to 15 mg/ml.

The nutritional composition may additionally comprise one or more ingredients selected from the group consisting of:

i. carbohydrate such as lactose and/or Inositol
ii. protein, preferably plant-based protein such as algae protein, pea protein or rapeseed protein, a portion of the protein my by hydrolyzed protein;

iii. long chain polyunsaturated fatty acids (LCPUFAs), such as docosahexaenoic acid (DHA) and/or arachidonic acid (ARA);
iv. vitamins, such as one or more of vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, Thiamin (vitamin B1), riboflavin (vitamin B2), nicotinic acid (vitamin B3), vitamin B6, vitamin B12, folic acid (vitamin B9), pantothenic acid (vitamin B5) and/or choline;
v. salts, such as one or more of calcium, phosphorus, magnesium, iron, zinc, manganese, copper, iodine, selenium, sodium, potassium, and/or chloride;
vi. enzymes or functional proteins, such as one or more of biotin, coenzyme Q10, and/or lactoferrin; and
vii. antioxidants, such as one or more of resveratrol, α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein, zeaxanthin, retinol and/or α-tocopherol.

Lactoferrin is a multifunctional protein of the transferrin family. Lactoferrin is a globular glycoprotein with a molecular mass of about 80 kDa. Lactoferrin is one of the transferrin proteins that transfer iron to the cells and control the level of free iron in the blood and external secretions. Lactoferrin has a very high isoelectric point (~pI 9) and its cationic nature plays a major role in its ability to defend against bacterial, viral, and fungal pathogens. Lactoferrin for use in the present disclosure may be, for example, isolated from the milk of a non-human animal or produced by a genetically modified organism. The oral electrolyte solutions described herein may comprise non-human lactoferrin, non-human lactoferrin produced by a genetically modified organism and/or human lactoferrin produced by a genetically modified organism.

In one embodiment the MFGM and HMO containing composition of the present disclosure does not contain lactoferrin.

Osteopontin is a highly glycosylated and phosphorus-acidified protein that is relatively high in breast milk (140 mg/L), and is known as milk protein, which is thought to mediate the cognitive, gut and immune system development of newborns.

In one embodiment the MFGM and HMO containing composition of the present disclosure does not contain osteopontin.

**Use of the compositions**

The present disclosure relates to one or more methods of providing a health benefit. The health benefit may be a non-medical benefit, but can also relate to treating a condition, disease, or disorder. The method of providing the health benefit is obtained by administering a composition comprising milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein said mixture of HMOs comprises a) at least one fucosylated HMO, and b) at least one HMO selected from of a neutral core HMO and/or a sialylated HMO to a subject. Preferably, if 6'-sialyllactose (6'-SL) is present in the HMO mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1, such as above 5:1, such as between 4-10:1, such as

between 4.5-8:1, such as between 5.5-7:1. Even more preferably at least one fucosylated HMO is 2'FL and if 6'-sialyllactose (6'-SL) is present in the mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1 , such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1. The amount of the composition that is administered is preferably in the range of 1 to 15 g/day of MFGM and 1 to 20 g/day of the HMO mixture. Preferably, the composition disclosed herein is administered in an amount which is effective (effective amount) to achieve the desired health benefit. Effectiveness can be assessed by comparing to the subject prior to treatment or to a non-administered control subject.

Furthermore, the present disclosure provides a non-medical use of the composition described herein. In particular, such non-medical use can be in a dietary supplement or infant formula or a growing up milk or food supplement.

An aspect of the present disclosure is a composition as described herein, such as a composition comprising milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein said mixture of HMOs comprises a) at least one fucosylated HMO, and b) at least one HMO selected from of a neutral core HMO and/or a sialylated HMO, for the use in supporting or improving one or more of the following:

a. brain health, and/or
b. immunity, and/or
c. recovery time following exercise,

in a subject. Preferably, if 6'-sialyllactose (6'-SL) is present in the HMO mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1, such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1. Even more preferably at least one fucosylated HMO is 2'FL and if 6'-sialyllactose (6'-SL) is present in the mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1 , such as above 5:1, such as between 4-10:1, such as between 4.5-8:1, such as between 5.5-7:1.

An embodiment is the use (non-medical) of a composition of the present disclosure in supporting or improving brain health which in particular relates to improving cognition, the ability to learn (learning) and memory in a subject. Preferably, the subject is an infant or an elderly. In an infant the use of the composition of the present disclosure promotes brain development by supporting the proliferation, migration, differentiation and survival of neural progenitor cells as well as facilitating neuronal activity and communication between brain cells and the formation of new neural connections.

An embodiment is the use, in particular non-medical use, of a composition of the present disclosure in maintaining the health of the intestinal lining, thereby supporting gut health as well as a healthy immune system.

The composition of the present disclosure may also be used in reduction of inflammation or symptoms thereof, in particular in a subject in need thereof. Thereby supporting a healthy immune system.

The composition of the present disclosure may also be used in the prevention or reduction of symptoms related to irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), Preferably, the composition of the present disclosure is administered in an effective dose to a subject in need thereof.

An embodiment is the use (non-medical) of a composition of the present disclosure in improving exercise performance by reducing muscle fatigue and improving recovery time after exercise. The composition of the present disclosure may be particularly useful for athletes with a high physical activity level. The composition may be a sports nutrition.

In embodiments, the use (non-medical or therapeutic) of the composition of the present disclosure is to increase the glutamic acid abundance/concentration/formation in the gut of the subject. The increase is preferably assessed relative to the level of glutamic acid in the gut prior to the administration of the composition of the present disclosure. As an alternative to measuring the glutamic acid in the gut of a subject the ability of a subject to produce glutamic acid following administration of the composition of the present disclosure can be accessed by fermenting a stool sample of said subject according to the method of example 1.

In certain embodiments a "subject" may be a human or a mammal, or other domestic animals such as pets (cats, dogs, rodents, rabbits, avian species, reptiles, etc.), livestock and performance animals (pigs, poultry, goat, sheep and cows) and working animals (horses, oxen, camels, donkeys and elephants) with a gut microbiome. Preferably, the subject is a human, such as an infant or a non-infant human. The term “infant human” or “infant” means a human of less than 3 years of age, a toddler is an infant of 1 to 3 years of age. The infant may be a pre-term infant, meaning that it is delivered before 37 weeks of pregnancy. The infant may be delivered by C-section, which means it has not been exposed to the natural microbiota in the vagina of the woman. The term “non-infant human” or “non-infant” means a human of 3 years of age and older. A non-infant human can be a child (age 3 to 12), a teenager (age 13 to 19), an adult or an elderly. The term "elderly" in the context of a human means an age from birth of at least 60 years, preferably above 65 years, more preferably above 70 years. The term "older adult" in the context of a human means an age from birth of at least 40 years, preferably above 50 years, more preferably above 55 years, and includes elderly individuals.

The terms “treat” or “treatment” or “treating” as used herein refers to both treatment of an existing disease (*e.g.,* a disease, condition or disorder as herein referred to) or prevention of a disease, *i.e.,* prophylaxis. Maintenance and/or promotion of health in an individual not suffering

from a disease, but who may be susceptible to the development of an unhealthy condition is considered non-medical treatment in the context of the present invention.

An "effective amount" of a composition of the present disclosure means an amount that render a desired health benefit/treatment outcome in the subject it is administered to. An effective amount can be administered in one or more doses to achieve the desired treatment outcome.

Preferably the effect of the use of the composition of the present disclosure is assessed after administration of a daily dose of the composition of the present disclosure for at least a week, such as at least 2 weeks, such as at least 3 weeks.

“Enteral administration” means any conventional form for delivery of a composition to a subject 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, direct delivery to the gut, sublingual and rectal.

"Oral administration" means any conventional form for the delivery of a composition to a non-infant through the mouth. Accordingly, oral administration is a form of enteral administration.

The proper dosage of a combination of the present disclosure may be determined, at least in part, based upon factors such immune status, body weight and age. In some cases, the dosage of the mixture of HMO will be similar to that found for the specific HMO components (e.g., 2’FL, DFL, 3FL, LNT, LNnT, 3’SL, 6’SL) in human breast milk. The recommended amount of the HMO mixture would generally be in the range from about 1 g to about 20 g per day, in certain embodiments from about 2 g to about 15 g per day, from about 3 g to about 10 g per day, in certain embodiments from about 1 g to about 10 g per day. Appropriate dose regimes can be determined based on the present disclosure and/or on factors known to a person of ordinary skill in the art. The dosage of the MFGM component of the composition disclosed herein would generally be in the range from about 1 g to about 15 g per day, in certain embodiments from about 1.5 g to about 10 g per day, from about 2 g to about 5 g per day, in certain embodiments from about 1 g to about 7 g per day.

***EXAMPLES***

***Materials***

The mixtures in Table 1 below were prepared using the following products

2'FL: GlyCare 2FL 9000
2'FL/DFL: GlyCare 2FL/DFL 8001
3FL: Under development by dsm-firmenich
LNnT: GlyCare LNnT 9000
LNT: GlyCare LNT 8001
3'SL: GlyCare 3SL 9001
6'SL: GlyCare 6SL 9001
MFGM: SureStart™ MFGM LIPID 70
GOS: Can for example be obtained from BOC Sciences (Cat. NO.: B1999-003025) or Biosynth (OG32134) or SureStart GOS from NZMP
PDX: Sigma 68424-04-4

***Example 1 – Glutamic acid***

In the present example, the effect of MFGM on mixtures of HMOs were tested.

A series of MFGM+HMO mixtures containing at least one fucosylated HMO and at least one neutral core HMO or a sialylated HMO (test compositions) were compared the same HMO mixtures without MFGM.

The Mixtures are shown in table 1.

Table 1: mixtures used in the present example, the amounts of the individual ingredients are in g/L of final assay volume

| Ref | Mixture | MFGM | 2'FL | DFL | LNnT | 6'SL | 3'SL | LNT | 3FL |
|---|---|---|---|---|---|---|---|---|---|
| 1 | MFGM | 2 | | | | | | | |
| 2 | MFGM+2'FL/DFL+LNnT+ 6'SL+ 3'SL | 2 | 2.28 | 0.29 | 0.37 | 0.4 | 0.19 | | |
| 3 | 2'FL/DFL+LNnT+ 6'SL+ 3'SL | | 2.28 | 0.29 | 0.37 | 0.4 | 0.19 | | |
| 4 | MFGM+2'FL+LNnT+ 6'SL+3'SL | 2 | 2.28 | | 0.37 | 0.4 | 0.19 | | |
| 5 | 2'FL+LNnT+ 6'SL+3'SL | | 2.28 | | 0.37 | 0.4 | 0.19 | | |
| 6 | MFGM+2'FL/DFL+ 6'SL+ 3'SL | 2 | 2.28 | 0.29 | | 0.4 | 0.19 | | |
| 7 | 2'FL/DFL+ 6'SL+ 3'SL | | 2.28 | 0.29 | | 0.4 | 0.19 | | |
| 8 | MFGM+2'FL/DFL+LNT+ 6'SL+ 3'SL | 2 | 2.28 | 0.29 | | 0.4 | 0.19 | 0.74 | |
| 9 | 2'FL/DFL+LNT+ 6'SL+ 3'SL | | 2.28 | 0.29 | | 0.4 | 0.19 | 0.74 | |
| 10 | MFGM+2'FL+3FL+LNT+ 6'SL+ 3'SL | 2 | 2.28 | | | 0.4 | 0.19 | 0.74 | 0.72 |
| 11 | 2'FL+3FL+LNT+ 6'SL+ 3'SL | | 2.28 | | | 0.4 | 0.19 | 0.74 | 0.72 |
| 12 | MFGM+2´FL+LNT+6'SL | 2 | 2.28 | | | 0.4 | | 0.74 | |
| 13 | 2´FL+LNT+6'SL | | 2.28 | | | 0.4 | | 0.74 | |
| 14 | MFGM+2´FL+LNnT+6'SL | 2 | 2.28 | | 0.37 | 0.4 | | | |
| 15 | 2´FL+LNnT+6'SL | | 2.28 | | 0.37 | 0.4 | | | |
| 16 | MFGM + 2'FL+LNT | 2 | 2.28 | | | | | 0.74 | |
| 17 | 2'FL+LNT | | 2.28 | | | | | 0.74 | |
| 18 | MFGM + 2'FL+LNnT | 2 | 2.28 | | 0.37 | | | | |
| 19 | 2'FL+LNnT | | 2.28 | | 0.37 | | | | |

The experimental setup was as follows. Feces samples were obtained from infant donors (age 2 to 5 month) (n = 5). Samples were prepared into 10% slurries using PBS and glycerol. The slurries were distributed in 48 well multi well plates (MTP), into Prodigest minimal nutrient media, in addition two control samples were added to each plate, one with the fecal slurries without test mixture (no_sugar) and one with media only. The fecal samples were subjected to the test mixtures in table 1 (n=2 x 5 donors) in a final volume of 0.6 mL and grown in the BioLector XT at 37°C for up 24 hours, shaking at 600 rpm under anaerobic conditions and continuous nitrogen gas flow. Samples were transferred to a 96 deep-well plate, spun down at 4000 rpm for at least 10 min at 4°C, and supernatants removed for analysis or stored at -80°C until further analysis.

Sample supernatants were analyzed in-house by Liquid Chromatography Mass Spectrometry (LC-MS) metabolomics.

To enable comparison of metabolite profiles across analytical runs, all data were center-scaled. This normalization technique adjusts the data such that the mean value becomes zero, mitigating any bias introduced by variations in sample amount. Additionally, for each Biolector run, the concentration of metabolites measured in the base media was subtracted from the concentration measured in all experiments within that run. This step removes background signal originating from the media itself, allowing for the direct comparison of metabolite production by the cells across different experiments.

*Results*

The data are shown in Figure 2.

From the data it can be seen that MFGM (01) alone does not have any effect on glutamic acid formation compared to the control without sugar. For the samples containing HMO mixtures these were tested in pairs, so one with MFGM (even numbers) and one without MFGM (uneven numbers). From this it can be seen that all the HMO mixtures without MFGM had a negative impact on the formation of glutamic acid compared to the No_sugar sample. Interestingly all the samples with MFGM and a mixture om HMOs containing 2'FL and at least one neutral core HMO and/or a sialylated HMO showed an increase in the formation of glutamic acid compared to the No_sugar control. This is highly surprising and clearly a synergistic effect since the individual components of the test mixes (MFGM or HMO mix) does not show any benefit, so combining them could not be expected to produce increased levels of glutamic acid. In particular for the mixture containing MFGM+2'FL/DFL+ 6'SL+ 3'SL (06) the difference in the effect on glutamic acid to the HMO mixture without MFGM (07) is substantial.

***CLAIMS***

1. A composition comprising milk fat globule membrane (MFGM) and a mixture of human milk oligosaccharides (HMOs), wherein said mixture of HMOs comprises:
   a. at least one fucosylated HMO, and
   b. at least one HMO selected from of a neutral core HMOs and/or a sialylated HMO, wherein, if 6'-sialyllactose (6'-SL) is present in the mixture, the ratio (w/w) of the fucosylated HMO:6'-SL is above 4:1.
2. The composition according to claim 1, wherein the fucosylated HMO is selected from the group of 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), difucosyllactose (DFL), lacto-N-fucopentaose I (LNFP-I) and lacto-N-difucohexaose I (LNDFH-I), or a mixture thereof.
3. The composition according to claim 1 or 2, wherein the fucosylated HMO(s) constitute at least 35 wt% of the mixture of HMOs.
4. The composition according to anyone of the preceding claims, wherein the neutral core HMO is selected from the group of lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) or a mixture thereof.
5. The composition according to anyone of the preceding claims wherein the sialylated HMO is selected from 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) or a mixture thereof.
6. The composition according to anyone of the preceding claims, wherein the composition comprises at least 2'FL, preferably both of 2'-FL and DFL.
7. The composition according to anyone of the preceding claims wherein the composition further comprises 3'-SL or 6'SL or both.
8. The composition according to anyone of the preceding claims, wherein the composition comprises either LNT or LNnT or both.
9. The composition according to any one of the preceding claims, wherein the mixture of HMOs is selected from one of the following mixtures:
   a. 2'FL and LNT, or
   b. 2'FL and LNnT, or
   c. 2'FL, LNT, 6'SL, or
   d. 2'FL, LNnT and 6'SL, or
   e. 2'FL, LNnT 6'SL and 3'SL, or
   f. 2'FL, DFL, LNnT, 6'SL and 3'SL, or
   g. 2'FL, DFL, LNT, 6'SL and 3'SL, or
   h. 2'FL, 3FL, LNT, 6'SL and 3'SL, or
   i. 2'FL, DFL, 3'SL and 6'SL, and

wherein the selected mixture of HMOs constitutes at least 95 wt% of total amount of HMOs in the composition.

10. The composition according to claim 1 or 9, wherein the mixture of HMOs consists essentially of
    a. 2'FL and LNT, or
    b. 2'FL and LNnT, or
    c. 2'FL, LNT, 6'SL, or
    d. 2'FL, LNnT and 6'SL, or
    e. 2'FL, LNnT 6'SL and 3'SL, or
    f. 2'FL, DFL, LNnT, 6'SL and 3'SL, or
    g. 2'FL, DFL, LNT, 6'SL and 3'SL, or
    h. 2'FL, 3FL, LNT, 6'SL and 3'SL, or
    i. 2'FL, DFL, 3'SL and 6'SL.
11. The composition according to claim 9 or 10, wherein the mixture of HMOs comprises or consists of 2'FL, DFL, 3'SL and 6'SL.
12. The composition according to anyone according to the preceding claims, wherein the composition is nutritional composition, such as an infant formula or growing up milk.
13. The nutritional composition according to claim 12, wherein the MFGM is added in an amount of 1 to 10 mg/ml and the HMO mixture is added in an amount of 1 to 15 mg/ml.
14. A composition according to anyone of claim 1 to 13, for the use in supporting or improving one or more of the following:
    a. brain health, such as improving cognition, learning and/or memory, and/or
    b. immunity, such as reduced inflammation, including reduced symptoms of irritable, bowel syndrome (IBS) and/or inflammatory bowel disease (IBD), and/or
    c. recovery time following exercise, including reducing muscle fatigue,

    in a subject.
15. The composition for the use according to claim 14, wherein the improvement of brain health and/or immunity and/or recovery time is based on an increase of glutamate production (concentration) in the gut.
16. The composition for the use according to claim 14 or 15, wherein the improvement of immunity encompasses the activation and regulation of immune cells, the contribution of the body's defense against pathogens and the maintenance of immune homeostasis.
17. The composition for the use according to 14 to 16, wherein the subject is an infant, child, older adult or elderly individual.