

Spray-dried lacto-*N*-fucopentaose

- 5 The present invention relates to preparations of human milk oligosaccharide. More specifically, the present invention relates to solid preparations of human milk oligosaccharides and to methods for the manufacturing of said solid preparations of human milk oligosaccharides.

Background

- 10 Human breast milk contains a substantial amount of carbohydrates. These carbohydrates include monosaccharides such as L-fucose and *N*-acetylneuraminic acid (Neu5Ac). The disaccharide lactose is also present in human breast milk. In addition to lactose, one liter of human breast milk contains up to 20 g/L oligosaccharides, the so-called "human milk oligosaccharides (HMOs)". HMOs represent the third most
- 15 abundant constituent of human breast milk. It is presumed that more than 150 structurally distinct oligosaccharides are present in human milk. Human milk usually contains between 10 and 13 major HMOs which are present in a concentration of between grams to several hundred milligrams per liter (Thurl et al., (2017), Nutrition Reviews 75(11) 920-933). HMOs include neutral HMOs as well as acidic HMOs
- 20 which contain one or more sialic acid moieties. The most prominent HMOs are shown in Table 1. The structural complexity and abundance of these oligosaccharides is unique for human milk and has not been found in the milk of other mammals such as – for example – domesticated dairy animals.

- Since HMOs are not digested by humans, the physiological role of these saccha-
- 25 rides is under investigation for several decades. The prebiotic effect of HMOs has been discovered over 100 years ago. HMOs are able to modulate the human gut microbiome by feeding beneficial bacteria. Several other functional effects of HMOs were investigated in the last years, especially their effect on neonates' development. HMOs are known to act as decoys to reduce the risk of infections by bacterial and
- 30 viral pathogens, which adhere to human cells by binding to cell surface glycoproteins. Additionally, various HMOs possess an anti-inflammatory effect and act as

immunomodulators. Hence, it was proposed that HMOs reduce the risks of developing food allergies. A positive effect of sialylated HMOs on neonatal brain development is intensely discussed (reviewed in "Prebiotics and Probiotics in Human Milk, Origins and functions of milk-borne oligosaccharides and bacteria", Academic Press
5 (2017) editors: McGuire M., McGuire M., and Bode L.).

HMOs include several structurally distinct lactofucopentaoses. The best characterized lactofucopentaoses are

lacto-*N*-fucopentaose I (LNFPI; $\text{Fuc}(\alpha 1,2)\text{Gal}(\beta 1,3)\text{GlcNAc}(\beta 1,3)\text{Gal}(\beta 1,4)\text{Glc}$),
lacto-*N*-fucopentaose II (NPFII; $\text{Gal}(\beta 1,3)(\text{Fuc}(\alpha 1,4))\text{GlcNAc}(\beta 1,3)\text{Gal}(\beta 1,4)\text{Glc}$),
10 lacto-*N*-fucopentaose III (LNFPIII; $\text{Gal}(\beta 1,4)(\text{Fuc}(\alpha 1,3))\text{GlcNAc}(\beta 1,3)\text{Gal}(\beta 1,4)\text{Glc}$),
and
lacto-*N*-fucopentaose V (LNFPV; $\text{Gal}(\beta 1,3)\text{GlcNAc}(\beta 1,3)\text{Gal}(\beta 1,4)(\text{Fuc}(\alpha 1,3))\text{Glc}$).

A first step to take advantage of the beneficial effects of HMOs for bottle-fed infants is the addition of individual HMOs to infant formula. However, supplementing
15 infant formulae with a combination of structurally distinct HMOs would be better, because a combination of structurally distinct HMOs will have effects that are more similar to the effects of their original source, human milk, and which can not be caused by individual HMOs.

The limited supply of individual HMOs for supplementing infant formulae has first led
20 to the development of chemical syntheses of HMOs, followed by biocatalytic approaches using purified enzymes. Today, fermentation of genetically-engineered bacterial cells is used to produce different HMOs in commercial scales (WO 2015/150328 A1, WO 2017/043382 A1, WO 2010/070104 A1, WO 2012/097950 A1).
The HMOs that are synthesized by the bacterial cells can be purified from the fermentation broth or cell lysate to obtain substantially pure preparations of the HMOs
25 such that they can be used in human food, especially in infant food.

During their purification, the lacto-*N*-fucopentaose is present in form of a liquid process stream. Along with the purification, the concentration of lacto-*N*-fucopentaose in the process stream is increased. However, an aqueous solution of a lacto-*N*-fucopentaose is very vulnerable for bacterial or fungal contamination. Therefore, it is
30

preferred to provide the lacto-*N*-fucopentaose as a dry product having a low content of water such that microbial growth is impossible.

Typically, a saccharide is obtained in solid form by crystallization. Crystallization of individual HMOs has been described: for 3-fucosyllactose (WO 2014/075680 A), for
5 2'-fucosyllactose (WO 2011/150939 A), Di-fucosyllactose (WO 2016/086947 A),
lacto-*N*-tetraose (WO 2017/101953 A), lacto-*N*-neotetraose (WO 2014/094783 A).
Crystallization of HMOs involves the use of organic solvents such as alcohols,
mainly ethanol or methanol, or organic acids such as glacial acetic acid. However,
the use of organic solvents for crystallizing HMOs as last step in the process of ob-
10 taining the final product in solid form is not appropriate if the HMOs shall be used as
food ingredients. In addition, organic solvents are harmful to the environment and to
any individual handling them. Thus, the use of organic solvents requires occupa-
tional safety measures and appropriate disposal which renders the use of organic
solvents to be costly. Therefore, crystallization of HMOs to provide the HMOs in
15 solid form has to be considered a drawback in the production of HMOs in an indus-
trial scale.

Therefore, a process is desired which provides HMOs, in particular lacto-*N*-fucopen-
taoses, in solid form, which is applicable in industrial scale production of HMOs, and
which does not involve the use of an organic solvent at the end of the purification
20 scheme to provide a solid preparation of said HMO.

The problem is solved by a process for providing a powder consisting essentially of
the purified HMO, wherein said method comprises spray-drying of an aqueous solu-
tion which contains the HMO.

Summary

25 In a first aspect, a spray-dried powder is provided method is provided consisting es-
sentially of at least one lacto-*N*-fucopentaose.

In a second aspect, a process for the manufacture of a spray-dried powder consist-
ing essentially of at least one lacto-*N*-fucopentaose.

In a third aspect, the use of the spray-dried powder consisting essentially of at least one lacto-*N*-fucopentaose for the manufacture of a nutritional composition is provided.

In a fourth aspect, a nutritional composition containing the spray-dried powder consisting essentially of at least one lacto-*N*-fucopentaose is provided.

Description of the figure

Fig. 1 shows a graph illustrating the results of a X-ray powder diffraction of spray-dried 3-fucosyllactose.

Fig. 2 shows a graph illustrating the results of a X-ray powder diffraction of spray-dried lacto-*N*-tetraose.

Fig. 3 shows a graph illustrating the results of a X-ray powder diffraction of spray-dried 6'-sialyllactose.

Fig. 4 shows a graph illustrating the results of a X-ray powder diffraction of spray-dried 3'-sialyllactose.

Fig. 5 shows a graph illustrating the results of a X-ray powder diffraction of a spray-dried mixture of 2'-fucosyllactose and lacto-*N*-tetraose.

Fig. 6 shows a graph illustrating the results of a X-ray powder diffraction of a spray-dried mixture of 2'-fucosyllactose, 3-fucosyllactose, lacto-*N*-tetraose, 3'-sialyllactose, and 6'-sialyllactose.

Detailed description

According to the first aspect, a spray-dried powder consisting essentially at least one lacto-*N*-fucopentaose is provided, wherein said lacto-*N*-fucopentaose has been produced by microbial fermentation.

The lacto-*N*-fucopentaose is produced by microbial fermentation as described herein below. The term "consisting essentially of" as used herein, means that the

spray-dried powder consists of at least one lacto-*N*-fucopentaose and – optionally – by-products that are generated during the microbial fermentation for the production of the at least one lacto-*N*-fucopentaose, but which could not have been removed from a process stream obtained from the microbial fermentation. The term “consist-
5 ing essentially of” includes spray-dried powders consisting of at least 80 %-wt., at least 85 %-wt., at least 90 %-wt., at least 93 %-wt., at least 95 %-wt. or at least 98 %-wt. lacto-*N*-fucopentaose.

In an additional and/or alternative embodiment, the at least one lacto-*N*-fucopentaose is selected from the group consisting of LNFPI, LNFPII, LNFPIII and LNFPIV.
10 In a particular embodiment, the lacto-*N*-fucopentaose is lacto-*N*-fucopentaose I. In an alternative embodiment, the spray-dried powder consists essentially of a mixture of lacto-*N*-fucopentaoses, wherein the lacto-*N*-fucopentaoses of the mixture are preferably selected from the group consisting of LNFPI, LNFPII, LNFPIII and LNFPIV.

15 In an additional and/or alternative embodiment, the lacto-*N*-fucopentaose is present in the spray-dried powder in amorphous form.

In an additional and/or alternative embodiment, the spray-dried powder contains $\leq$ 15 %-wt. of water, preferably $\leq$ 10 %-wt. of water, more preferably $\leq$ 7 %-wt. of water, most preferably $\leq$ 5 %-wt. of water.

20 In an additional and/or alternative embodiment, the spray-dried powder is free of genetically-engineered microorganisms and nucleic acid molecules derived from genetically-engineered microorganisms.

According to the second aspect, provided is a process for the manufacture of a spray-dried powder consisting essentially of at least one lacto-*N*-fucopentaose
25 which has been produced by microbial fermentation. The process comprises the steps of:

- a) purifying lacto-*N*-fucopentaose from a process stream;
- b) providing an aqueous solution of the at least one lacto-*N*-fucopentaose of step a); and
- 30 c) subjecting the solution of step b) to spray-drying.

In an additional and/or alternative embodiment, purifying the lacto-*N*-fucopentaose from a process stream includes one or more of the steps of:

- i) removing the microbial cells from the fermentation broth and/or lysing the microbial cells to obtain a process stream;
- 5 ii) subjecting the process stream to at least one ultrafiltration;
- iii) treating the process stream at least one time with a cation exchange resin and/or at least one time with an anion exchange resin;
- iv) subjecting the process stream to at least one nanofiltration;
- v) subjecting the process stream to at least one electrodialysis;
- 10 vi) treating the process stream at least one time with activated charcoal; and/or
- vii) subjecting the process stream at least one time to a crystallization and/or precipitation step.

The lacto-*N*-fucopentaose may be produced by microbial fermentation, wherein a
15 genetically-engineered microorganism that is able to synthesize the lacto-*N*-fucopentaose is cultivated in a culture medium (fermentation broth) and under conditions that are permissive for the synthesis of the lacto-*N*-fucopentaose by said genetically-engineered microorganism. The purification of lacto-*N*-fucopentaose produced by microbial fermentation comprises the step of separating the microbial
20 cells from the fermentation broth to obtain a cleared process stream which essentially free of cells and which contains the lacto-*N*-fucopentaose. This step is the first step in the process of purifying the desired oligosaccharides.

Suitable methods for separating the microbial cells from the fermentation broth include centrifugation wherein the microbial cells are obtained as a pellet and the
25 fermentation broth as a supernatant. In an additional and/or alternative embodiment, the microbial cells are separated from the fermentation broth by means of filtration. Suitable filtration methods for separating the cells from the fermentation broth include microfiltration and ultrafiltration.

Microfiltration as such is a physical filtration process where a particle-containing fluid
30 is passed through a special pore-sized membrane to separate the particles from the fluid. The term "microfiltration" as used herein refers to a physical filtration process where cells are separated from the fermentation broth.

Ultrafiltration is a variety of membrane filtration and is not fundamentally different. In ultrafiltration, forces like pressure or concentration gradients lead to a separation through a semipermeable membrane. Cells, suspended solids and solutes of high molecular weight are retained in the so-called retentate, while water and low
5 molecular weight solutes such as the desired sialylated oligosaccharide pass through the membrane in the permeate (filtrate).

Ultrafiltration membranes are defined by the molecular weight cut-off (MWCO) of the membrane used. Ultrafiltration is applied in cross-flow or dead-end mode.

10 In embodiments, wherein at least a portion of the lacto-*N*-fucopentaose which has been synthesized by the microbial cell is not secreted into the fermentation broth, but remains intracellularly, the cells may be removed from the fermentation broth and lysed. Insoluble constituents may be removed from the cell lysate which then becomes the process stream which contains the lacto-*N*-fucopentaose.

Notwithstanding that the process is used for the purification of lacto-*N*-fucopentaose
15 that has been produced by microbial fermentation, said process may also be employed to purify a lacto-*N*-fucopentaose that was produced by enzymatic catalysis *in-vitro*. The lacto-*N*-fucopentaose can be purified from the reaction mixture at the end of the biocatalytic reaction. Said reaction mixture is subjected to the process for the purification as process stream.

20 The process stream contains the lacto-*N*-fucopentaose as well as a by-products and undesired impurities such as – for example - monosaccharides, disaccharides, undesired oligosaccharide by-products, ions, amino acids, polypeptides, proteins and/or nucleic acids.

25 In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises the step of at least one cation exchange treatment to remove positively charged compounds from the cleared process stream.

Suitable cation exchange resins for removing positively charged compounds include Lewatit S2568 (H+) (Lanxess AG, Cologne, DE).

In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises the step of an anion exchange treatment to remove undesired negatively charged compounds from the cleared process stream.

Suitable anion exchange resins include Lewatit S6368 A, Lewatit S4268, Lewatit S5528, Lewatit S6368A (Lanxess AG, Cologne, DE), Dowex AG 1x2 (Mesh 200-400), Dowex 1x8 (Mesh 100-200), Purolite Chromalite CGA100x4 (Purolite GmbH, Ratingen, DE), Dow Amberlite FPA51 (Dow Chemicals, MI, USA).

In an additional/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises a nanofiltration and/or a diafiltration step to remove impurities having a lower molecular weight, and to concentrate the desired oligosaccharides.

Diafiltration involves the addition of fresh water to a solution in order to remove (wash out) membrane-permeable components. Diafiltration can be used to separate components on the basis of their molecular size and charge by using appropriate membranes, wherein one or more species are efficiently retained and other species are membrane permeable. In particular, diafiltration using a nanofiltration membrane is effective for the separation of low molecular weight compounds like small molecule and salts. Nanofiltration membranes usually have a molecular weight cut-off in the range 150 - 1000 Daltons. Nanofiltration is widely used in the dairy industry for the concentration and demineralization of whey.

Suitable membranes for nanofiltration and/or diafiltration include Dow Filmtec NF270-4040, Trisep 4040-XN45-TSF (Microdyn-Nadir GmbH, Wiesbaden, DE), GE4040F30 and GH4040F50 (GE Water & Process Technologies, Ratingen, DE).

Diafiltration using nanofiltration membranes was found to be efficient as a pretreatment to remove significant amounts of contaminants prior to electrodialysis treatment of the solution containing the oligosaccharide. The use of nanofiltration membranes for concentration and diafiltration during the purification of HMOs results in lower energy and processing costs, and better product quality due to reduced thermal exposure, leading to reduced Maillard reactions and aldol reactions.

In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises at least one electrodialysis step.

Electrodialysis (ED) combines dialysis and electrolysis and can be used for the separation or concentration of ions in solutions based on their selective electromigration
5 through semipermeable membranes.

The basic principle of electrodialysis consists of an electrolytic cell comprising a pair of electrodes submerged into an electrolyte for the conduction of ions, connected to a direct current generator. The electrode connected to the positive pole of the direct current generator is the anode, and the electrode connected to the negative pole is
10 the cathode. The electrolyte solution then supports the current flow, which results from the movement of negative and positive ions towards the anode and cathode, respectively. The membranes used for electrodialysis are essentially sheets of porous ion-exchange resins with negative or positive charge groups, and are therefore described as cationic or anionic membranes, respectively. The ion-exchange mem-
15 branes are usually made of polystyrene carrying a suitable functional group (such as sulfonic acid for cationic membranes or a quaternary ammonium group for anionic membranes) cross-linked with divinylbenzene. The electrolyte can be, for example, sodium chloride, sodium acetate, sodium propionate or sulfamic acid. The electrodialysis stack is then assembled in such a way that the anionic and cationic mem-
20 branes are parallel as in a filter press between two electrode blocks, such that the stream undergoing ion depletion is well separated from the stream undergoing ion enrichment (the two solutions are also referred to as the diluate (undergoing ion depletion) and concentrate (undergoing ion enrichment)). The heart of the electrodialysis process is the membrane stack, which consists of several anion-exchange
25 membranes and cation-exchange membranes separated by spacers, installed between two electrodes. By applying a direct electric current, anions and cations will migrate across the membranes towards the electrodes.

In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose further comprises a step of continuous chromatography like
30 simulated bed moving (SMB) chromatography.

Simulated moving bed (SMB) chromatography originated in the petrochemical and mineral industries. Today, SMB chromatography is used by the pharmaceutical industry to isolate enantiomers from racemic mixtures. Large-scale SMB chromatography has already been used for the separation of the monosaccharide fructose
5 from fructose-glucose solutions and for the separation of the disaccharide sucrose from sugar beet or sugar cane syrups.

SMB processes used to separate saccharides use e.g. calcium charged, cross-linked polystyrene resins, anion resins in the bisulfite form (Bechthold M., et al., Chemie Ingenieur Technik, 2010, 82, 65-75), or polystyrenic gel strong acid cation
10 resin in the hydrogen form (Purolite PCR833H) (Purolite, Bala Cynwyd, USA).

Given the continuous mode of operation, the recycling of the mobile phase and also the potential to use large column sizes, SMB systems can in principle be scaled to achieve production volumes of hundreds of tons.

The process step of simulated moving bed chromatography is advantageous in that
15 this process step allows further removal of oligosaccharides being structurally closely related to the desired oligosaccharide.

In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises a treatment of the process stream with activated charcoal to remove contaminating substances such as colorants from the process
20 stream.

In additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises at least one step of crystallization or precipitation of the lacto-*N*-fucopentaose from the process stream. Crystallization or precipitation of lacto-*N*-fucopentaose from the process stream may be performed by adding a suitable
25 amount of an organic solvent that is miscible with water to the process stream containing the lacto-*N*-fucopentaose. The organic solvent may be selected from the group consisting of C₁- to C₆-alcohols and C₁- to C₄-carbon acids.

In an additional and/or alternative embodiment of the process for the purification of the lacto-*N*-fucopentaose comprises a step sterile filtration and/or endotoxin removal, preferably by filtration of the process stream through a 3 kDa filter or 6 kDa filter.

- 5 In an additional and/or alternative embodiment, the process for the purification of lacto-*N*-fucopentaose comprises a step of increasing the concentration of lacto-*N*-fucopentaose in the process stream. The concentration of the lacto-*N*-fucopentaose in the process stream can be increased by subjecting the process stream to vacuum evaporation, reverse osmosis or nanofiltration (e.g. nanofiltration with a nanofiltration membrane having a size exclusion limit of $\leq 20 \text{ \AA}$). Alternatively, crystallized or
10 precipitated lacto-*N*-fucopentaose is dissolved in water, to obtain a solution of the lacto-*N*-fucopentaose possessing the desired concentration of the lacto-*N*-fucopentaose.

- In an additional and/or alternative embodiment, the resulting process stream is an
15 aqueous solution which contains the at least one lacto-*N*-fucopentaose in a concentration of $\geq 20 \text{ g/L}$, $\geq 25 \text{ g/L}$, $\geq 30 \text{ g/L}$, $\geq 40 \text{ g/L}$, $\geq 60 \text{ g/L}$, $\geq 100 \text{ g/L}$, $\geq 200 \text{ g/L}$ or even $\geq 300 \text{ g/L}$.

- In an additional and/or alternative embodiment, the aqueous solution contains the at least one lacto-*N*-fucopentaose in a purity of at least 80 %, at least 85 %, at least
20 90 %, at least 93 %, at least 95 % or at least 98 % with respect to the weight of dry matter/solutes within the solution.

The obtained concentrate containing the purified lacto-*N*-fucopentaose can be stored under appropriate conditions.

- The process for the purification of lacto-*N*-fucopentaose is cost efficient and easy to
25 scale up, making it suitable as a basis for a multi-ton scale manufacturing process.

- The process for the purification of lacto-*N*-fucopentaose is also advantageous in that the aqueous solution is free of genetically-engineered microorganisms and nucleic acid molecules derived from genetically-engineered microorganisms. In addition, the aqueous solution is free of proteins. The total removal of proteins eliminates the risk
30 of causing allergies to a potential consumer.

The process for the manufacture of the spray-dried powder comprises the step of providing an aqueous solution containing the at least one lacto-*N*-fucopentaose.

In an additional and/or alternative embodiment, the aqueous solution contains the at least one lacto-*N*-fucopentaose in an amount of at least 20 % (w/v), 30 % (w/v),
5 35 % (w/v), and up to 45 % (w/v), 50 % (w/v), 60 % (w/v).

In an additional and/or alternative embodiment, the aqueous solution contains the at least one lacto-*N*-fucopentaose in a purity of at least 80 %, at least 85 %, at least 90 %, at least 93 %, at least 95 % or at least 98 % with respect to the weight of dry matter/solutes within the solution.

10 In an additional and/or alternative embodiment, the aqueous solution does not contain genetically-engineered microorganisms, nucleic acid molecules derived from genetically-engineered microorganisms and proteins.

In the process of the manufacture of the spray-dried powder, the aqueous solution containing the at least one lacto-*N*-fucopentaose is subjected to spray-drying.

15 Spray-drying is a method to obtain dry powders, wherein the solution containing the substance of interest (i.e. lacto-*N*-fucopentaose) is first sprayed into droplets which are rapidly dried by hot air. Spray-drying is very fast and exposure of the substance to be dried to high temperatures is quite short.

In an additional and/or alternative embodiment, the aqueous solution containing the
20 at least one lacto-*N*-fucopentaose that has been purified from a fermentation broth or process stream is spray-dried at a nozzle temperature of at least 110 °C, preferably at least 120 °C, more preferably at least 125 °C, and less than 150 °C, preferably less than 140 °C and more preferably less than 135 °C.

In an additional and/or alternative embodiment, the aqueous solution containing the
25 at least one lacto-*N*-fucopentaose that has been purified from a fermentation broth or process stream is spray-dried at an outlet temperature of at least 60 °C, preferably at least 65 °C, and less than 80 °C, preferably less than 70 °C. In a particularly preferred embodiment, the aqueous solution containing the at least one lacto-*N*-fucopentaose is spray-dried at a nozzle temperature of about 68 °C to about 70 °C.

It is understood that any one of the structurally distinct lacto-*N*-fucopentaoses can be purified and spray-dried individually, and that the resulting spray-dried powders can be mixed in any desired ratio. In an additional and/or alternative embodiment, separate aqueous solutions each containing a separate structurally distinct lacto-*N*-fucopentaose can be mixed in any desired ratio, and the resulting aqueous solution contains a mixture of structurally distinct lacto-*N*-fucopentaoses in a desired ratio can be subjected to spray-drying. The ratio of the different lacto-*N*-fucopentaoses in the resulting spray-dried powder corresponds to the ratio of the different lacto-*N*-fucopentaose in the aqueous solution.

- 10 The spray-drying of the aqueous solution containing at least one lacto-*N*-fucopentaose provides a powder of low hygroscopy, wherein the lacto-*N*-fucopentaose is present in amorphous form, and wherein the particle size is homogeneous.

According to the third aspect, provided is the use of the spray-dried powder containing at least one lacto-*N*-fucopentaose that has been purified from a process stream for the manufacture of a nutritional composition. The spray-dried powder consisting essentially of at least one lacto-*N*-fucopentaose is suitable for human consumptions and may thus be included into preparations for human consumption such as medicinal formulations, infant formula, dairy drinks or dietary supplements.

- 20 According to the fourth aspect, provided are nutritional compositions which contain a spray-dried powder as described in the first aspect or as manufactured according to the second aspect.

In an additional and/or alternative embodiment, the nutritional composition contains at least one additional HMO which is not the at least one lacto-*N*-fucopentaose which is present in or as said spray-dried powder. The at least one additional HMO may be a neutral HMO, preferably selected from the group consisting of 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), and lacto-*N*-fucopentaose I (LNFPI), lacto-*N*-fucopentaose II (LNFPII), lacto-*N*-fucopentaose III (LNFPIII) and lacto-*N*-fucopentaose V (LNFPIV).

In an additional and/or alternative embodiment, the at least one additional HMO may be a sialylated HMO, preferably selected from the group consisting of 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), sialyllacto-*N*-tetraose (LST)-a, LST-b, LST-c and disialyllacto-*N*-tetraose (DSLNT).

- 5 In an additional and/or alternative embodiment, the nutritional composition includes a mixture consisting essentially of Neu5Ac, 2'-FL, 3-FL, LNT, LNnT, LNFPI, 3'-SL, 6'-SL, sialic acid and L-fucose. A nutritional composition including preferred amounts of each of said compounds is provided in Table 1.

Compound	Proportion in mix (percentage by weight)	Final concentra- tion in infant formula (g/L)
2'-FL	34	2.5
3-FL	11	0.8
LNT	20	1.5
LNnT	2	0.15
LNFPI	13	1.0
3'-SL	3	0.2
6'-SL	4	0.3
Neu5Ac	8	0.6
L-Fucose	5	0.4
Total	100	7.45

- 10 Table 1: Composition of an exemplary mixture containing suitable as supplement for infant formulae.

The composition according to the second column in Table 1 is of particular advantage for supplementing infant formula such that the final infant formula for direct consumption may contain the compounds of the mixture in concentrations as specified in the third column of Table 1.

- 15 In an additional and/or alternative embodiment, the nutritional composition contains one or more additional ingredients. Said one or more additional ingredients are selected from the group consisting of oil, fat and fatty acids (such as olive oil, sunflower oil, coconut oil, nut oil, rapeseed oil, palm oil, flaxseed oil, fish oil, linolenic acid, soy-bean oil, etc.), carbohydrates (such as glucose, fructose, lactose, malto-
20 dextrin, starch, sucrose, inositol, etc.) proteins (from skim milk, whey, casein

(derived from any domesticated dairy animals), or soy bean), vitamins (A, B1, B2, B5, B6, B12, C, D, E, K, biotin, folic acid, niacin, choline) minerals and trace elements (sodium, potassium, chloride, calcium, phosphorus, magnesium, iron, zinc, manganese, fluoride, selenium, iodine, copper).

- 5 In a preferred embodiment the nutritional composition containing the spray-dried human milk oligosaccharides or the mixture of human milk oligosaccharides or the mixture of human milk oligosaccharides with functional monosaccharides or the mixture of human milk oligosaccharides with other fibers is an infant formula that meets the compositional requirements set forth in Regulation (EU) 2016/127 and/or in the
- 10 Code of Federal Regulations (USA) Title 21 107.100 (nutrient specifications). Representative compositions of infant formulas are specified in Tables 2 and 3.

Infant formula:	Skimmed milk Vegetable oils (palm oil, rapeseed oil, sunflower oil) Human milk oligosaccharides LNFP1 Skimmed milk powder Oil of <i>Mortierella alpine</i> Fish oil Calcium carbonate Potassium chloride Vitamin C Sodium chloride Vitamin E Iron acetate Zinc sulfate Niacin Calcium-D-pantothenate Copper sulfate Vitamin A Vitamin B1 Vitamin B6 Magnesium sulfate Potassium iodate Folic acid Vitamin K Sodium selenite Vitamin D
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Table 2: Components of an exemplary infant formula.

		per 100 g powder	per 100 ml infant formula
Energy	kJ	2094–2145	283
	kcal	500–512	67–68
Fats, hereof:	g	24.2–26.2	3.3–3.5
saturated fatty acids	g	8.7–9.4	1.2–1.3
monounsaturated fatty acids	g	10.4	1.4
polyunsaturated fatty acids	g	5.5–5.9	0.7–0.8
Carbohydrates hereof:	g	56–58	7.4–7.9
Sugars hereof:	g	44–56	6–7.4
Lactose	g	44–56	6–7.4
Neu5Ac	mg	440	60
L-fucose	mg	300	40
HMOs hereof	g	4.22–4.81	0.57–0.65
2'-FL	g	1.85–2.22	0.25–0.30
3-FL	mg	555.56–592.6	75–80
LNT	g	1.11	0.15
LNnT	mg	0–111.11	0–15
LNPF-I	mg	0–740.74	0–100
3'-SL	mg	148.15–170.37	20–23
6'-SL	mg	207.4–222.22	28–30
Protein	g	11.11–11.85	1.5–1.6
Salt	g	0.47–0.59	0.06–0.08
Vitamins			
Vitamin A	µg	357–358	47.3–48.2
Vitamin D	µg	7.8	1.05
Vitamin E	mg	8.15	1.1
Vitamin K	µg	43.7–44.4	5.9–6.0
Vitamin C	mg	115–118	15–16
Vitamin B1	mg	0.51–0.60	0.068–0.079
Vitamin B2	mg	1.3–1.7	0.18–0.23
Niacin	mg	3.63	0.49
Vitamin B6	µg	526–600	71–81
Folic acid	µg	160–164	21.6–21.7
Vitamin B12	µg	1.7–1.9	0.23–0.25
Biotin	µg	22–30	3.0–3.9
Panthothenic acid	mg	4.6–5.4	0.62–0.72
Minerals			
Sodium	mg	187–236	25.3–31.2
Potassium	mg	673–675	88.8–91.2
Chloride	mg	327–333	43.1–44.9
Calcium	mg	460–504	62.1–66.5
Phosphorous	mg	335–352	45.2–46.5
Magnesium	mg	49.3–56.3	6.66–7.43
Iron	mg	4.15	0.56

		per 100 g powder	per 100 ml infant formula
Zinc	mg	3.7–3.8	0.49–0.51
Copper	µg	274	37
Manganese	µg	96.3	13
Fluoride	µg	30.4–32.6	4.1–4.4
Selenium	µg	11.1–12.3	1.5–1.6
Iodine	µg	101.5–103.7	13.7–14

Table 3: Composition of an exemplary infant formula. The final concentration is based on a preparation of 13.5 g of the powder in 90 ml of water.

In an additional and/or alternative embodiment, the nutritional composition also contains microorganisms, preferably probiotic microorganisms. For infant food applications, the preferred microorganisms are derived from or can be found in the microbiome of a healthy human. Preferably, but with no limitations, the microorganisms are selected from the genera *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Leuconostoc*, *Clostridium*, *Eubacterium*, *Veillonella*, *Fusobacterium*, *Bacterioides*, *Prevotella*, *Escherichia*, *Propionibacterium* and *Saccharomyces*. In an additional and/or alternative embodiment, the microorganism is selected from the group consisting of *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*; *Enterococcus faecium*; *Escherichia coli*; *Kluyveromyces marxianus*; *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*, *L. sakei*; *Lactococcus lactis* (including but not limited to the subspecies *lactis*, *cremoris* and *diacetylactis*); *Leuconostoc mesenteroides* (including but not limited to subspecies *mesenteroides*); *Pedococcus acidilactici*, *P. pentosaceus*; *Propionibacterium acidipropionici*, *P. freudenreichii* ssp. *shermanii*; *Staphylococcus carnosus*; and *Streptococcus thermophilus*.

In addition to the combination living organisms, the nutritional composition can also include dead cell cultures. In the field of probiotics, killed cell cultures are sometimes used (e.g. tyndalized bacteria). These killed cultures may provide proteins, peptides, oligosaccharides, cell outer wall fragments and natural products, leading to the short-term stimulation of the immune system.

Including probiotic microorganisms in the nutritional composition, especially in the presence of HMOs, is particularly advantageous in that it also promotes the establishment of a healthy gut microbiome.

5 In an additional and/or alternative embodiment, the nutritional composition also includes prebiotics such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin or combinations thereof.

The nutritional composition is present in solid form including, but not limited to, powders, granules, flakes, pellets or combinations thereof.

10 In an additional embodiment, the nutritional composition is selected from the group consisting of medicinal formulations, infant formulas, dairy drinks and dietary supplements.

As a medicinal formulation, the nutritional composition may be used to improve cognitive performance, especially for improving attention, learning and/or memory.

15 The present invention will be described with respect to particular embodiments and with reference to drawings, but the invention is not limited thereto but only by the claims. Furthermore, the terms first, second and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood that the terms so used are interchangeable under
20 appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

25 It is to be noticed that the term "comprising", as used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression "a device comprising means A and B" should not be limited to devices consisting only of components A

and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification do not necessarily refer always to the same embodiment. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly, it should be appreciated that in the description of representative embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and facilitating the understanding of one or more of the various inventive aspects. This method of disclosure is not to be interpreted as reflecting an intention that the claimed invention requires more features than are expressly listed in each claim. Rather, as the following claims reflect, inventive aspects may require fewer than all the features of any foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

Furthermore, whereas some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those familiar with the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor with the necessary instructions for carrying out such a method or element of a

method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

- 5 In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order to facilitate the understanding of the description and drawings.
- 10 The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art without departing from the true spirit or technical merit of the invention, the invention being limited only by the terms of the appended claims.

15 **Example 1: Purification of 2'-fucosyllactose from fermentation broth**

- Production of 2'-fucosyllactose by fermentation using a genetically modified *E. coli* strain was performed as described in European patent application No. 16 196 486.1. The 2'-fucosyllactose was purified from the fermentation broth by filtration, ion exchange chromatography, nanofiltration, diafiltration or electrodialysis, and treatment
- 20 with charcoal as described in WO 2015/106943 A1. The resulting solution containing 2'-fucosyllactose was subjected to spray-drying to obtain a stable solid product.

Example 2: Purification of 3-fucosyllactose from fermentation broth

- 3-Fucosyllactose was produced by fermentation using a genetically modified *E. coli* strain as described in European patent application No. 16 196 486.1.
- 25 The cells were separated from the culture medium by ultrafiltration (0.05 µm cut-off) (CUT membrane technology, Erkrath, Germany) followed by a cross-flow filter with a MWCO of 150 kDa (Microdyn-Nadir, Wiesbaden, Germany). The cell-free fermentation medium containing about 30 g/L 3-fucosyllactose, was passed over a strong

cationic ion exchanger (Lewatit S 2568 (Lanxess, Cologne, Germany) in H⁺ form to remove positive charged contaminants. Afterwards the solution was set to pH 7.0 using sodium hydroxide and applied to an anionic ion exchanger (Lewatit S6368 A, Lanxess) in the chloride form. Both ion exchangers were used in 200 L volume. After a second filtration (150 kDa; Microdyn-Nadir, Wiesbaden, Germany) the particle free solution was concentrated 5-fold by nanofiltration using a Filmtech NF270 membrane (Dow, Midland, USA) and 2.5-fold by vacuum evaporation. The concentrated solution and a conductivity of about 15 mS cm⁻¹ was filtrated (10 kDa; Microdyn-Nadir, Wiesbaden, Germany), clarified by activated carbon charcoal (CAS:7440-44-0, Carl Roth, Karlsruhe, Germany) and deionized by electrodialysis. Therefor a PC-Cell BED 1-3 electrodialysis apparatus (PC-Cell, Heusweiler, Germany) with a PC-Cell E200 membrane stack was used containing the following membranes: cation exchange membrane CEM:PC SK and anion membrane AEM:PCAcid60. 0.25 M Sulphamic acid was used as electrolyte in the process. For reduction of brownish coloring caused by Maillard-reactions and aldol products originating from the fermentation process, a second round of ion exchange chromatography was performed using the same ion exchange material as aforementioned in Na⁺ and Cl⁻ form, however in a volume of 50 L. After concentrating the sugar solution by evaporation, again the conductivity was reduced from 4 mS cm⁻¹ to 0.4 mS cm⁻¹ or less by electrodialysis using the PC-Cell BED 1-3 mentioned previously. For further decolorization the solution was mixed with activated charcoal (CAS:7440-44-0, Carl Roth, Karlsruhe, Germany) and a nearly colorless solution was obtained by filtration.

Example 3: Purification of lacto-*N*-tetraose from fermentation broth

Fermentative production of Lacto-*N*-tetraose was conducted using a genetically modified *E. coli* BL21 (DE3) Δ *lacZ* strain, with genomically integrated genes essential for the *in vivo* synthesis of Lacto-*N*-tetraose., namely, a *N*-acetylglucosamine glycosyltransferase (*lgtA* from *Neisseria meningitidis* MC58), a β -1,3-galactosyltransferases (*wbdO* from *Salmonella enterica* subsp. *salamae* serovar Greenside), *lacY* from *E. coli* K12, the UDP-glucose-4-epimerase *gaIE*, and the UTP-glucose-1-phosphat uridylyltransferase *gaIJ*, both from *E. coli* K12. In addition, the *gaS* gene encoding a glucosamine-6-phosphate synthase was overexpressed. For the fermentative production of Lacto-*N*-tetraose the strain was grown in a defined mineral

salts medium comprising 2% glucose as carbon source. Antifoam was added when needed. The pH was controlled using a 25% ammonia solution. Lactose was added stepwise to a final concentration of 15 mM from a 216 g l⁻¹ lactose stock, the lactose concentration in the culture medium was held constant throw-out the fermentation process. Residual lactose and Lacto-*N*-triose II, accumulation during the process as by-product, was hydrolyzed by a second *E. coli* strain that was added to the fermenter. This strain expressed a functional beta-lactamase, a beta-*N*-acetylhexosaminidase (*bbhI* from *Bifidobacterium bifidum* JCM1254), and a functional gal-operon for degradation of monosaccharides (EP 2 845 905 A).

- 10 Cells were separated from the fermentation broth, and the lacto-*N*-tetraose containing fluid was purified to a purity of 75-80 %, determined by mass balance, according to the procedure described in example 2.

Contaminating carbohydrate by-products resulting from inefficient enzymatic degradation and metabolization were removed by chromatography using a simulated moving bed (SMB) chromatography, according to WO 2015/049331. Alternatively, the lacto-*N*-tetraose was purified by crystallization with isopropanol. For crystallization the lacto-*N*-tetraose containing solution was concentrated by evaporation to a concentration of 20% and spray-dried. Using a NUBILOSA LTC-GMP spray dryer (NUBILOSA, Konstanz, Germany) the solution was passed under nitrogen flow through the spray dryers nozzles with an inlet temperature of 130°C while the product flow was controlled to maintain an outlet temperature of 67°C to 68°C.

The solid material was added to a mixture of isopropanol and water (3:1 (vol/vol)) in a ratio of 1 kg powder in 12 L isopropanol/water. The suspension was stirred vigorously, then the insoluble lacto-*N*-tetraose was filtrated and dried at 40 °C. Starting with a 73-89 % pure material, the crystallized Lacto-*N*-tetraose was purified to about 95 %, with a recovery of 85 %. The sugar was dissolved in water to a concentration of 25 % and passed sequentially through a 6 kDa filter (Pall Microza ultrafiltration module SIP-2013, Pall Corporation, Dreieich, Germany) and a 0.2 µm sterile filter. Solid material was obtained by spray drying the sterile material under the conditions described above.

Example 4: Purification of 3'- and 6'-Sialyllactose from fermentation broth

For production of 3'- sialyllactose and 6'-sialyllactose recombinant *E. coli* BL21 (DE3) $\Delta lacZ$ strains were used. The strains had common genetic modifications: chromosomal, constitutive expression of the glucosamine-6-phosphate synthase
5 GlmS from *E. coli*, the *N*-acetylglucosamin-2-epimerase Slr1975 from *Synechocystis* sp., the glucosamine 6-phosphat *N*-acetyltransferase Gna1 from *Saccharomyces cerevisiae*, the phosphoenolpyruvate synthase PpsA from *E. coli*, the *N*-acetylneuramate synthase NeuB, and the CMP-sialic acid synthetase NeuA, the latter both from *Campylobacter jejuni*. Additionally, the genes encoding the lactose permease
10 LacY from *E. coli*, *cscB* (sucrose permease), *cscK* (fructokinase), *cscA* (sucrose hydrolase), and *cscR* (transcriptional regulator) from *E. coli* W, and a functional *gal*-operon, consisting of the genes *galE* (UDP-glucose-4-epimerase), *galT* (galactose-1-phosphate uridylyltransferase), *galK* (galactokinase), and *galM* (galactose-1-epimerase) from *E. coli* K12 were integrated into the genome of the BL21 strain, and
15 constitutively expressed.

The strain synthesizing 3'-sialyllactose harbors the alpha-2,3-sialyltransferase gene from *Vibrio* sp. JT-FAJ-16, while the 6'-sialyllactose producing strain contains the alpha-2,6-sialyltransferase plsT6 from *Photobacterium leiognathi* JT-SHIZ-119.

The sialyllactose producing strains were grown in a defined mineral salts medium
20 containing 2% sucrose as carbon source. The sucrose feed (500 g l⁻¹), fed in the fed-batch phase, was supplemented with 8 mM MgSO₄, 0.1 mM CaCl₂, trace elements, and 5 g l⁻¹ NH₄Cl.

For sialyllactose formation, a lactose feed of 216 g l⁻¹ was employed. The pH was controlled by using ammonia solution (25% v/v). Fed batch fermentation was conducted at 30°C under constant aeration and agitation. In order to remove residual
25 lactose at the end of the fermentation, β -galactosidase was added to the fermentation vessel. The resulting monosaccharides were metabolized by the production strain.

The cell-free liquid was then deionized by ion exchange chromatography. First, cationic contaminants were removed on a strong cationic exchanger in a volume of 200
30 L (Lewatit S 2568 (Lanxess, Cologne, Germany) in H⁺ form. Using NaOH the pH of

the obtained solution was set to 7.0. In a second step, anionic ions and undesired colorants were removed from the solution using the strong anionic exchanger Lewatit S 6368 S (Lanxess, Cologne, Germany) in the chloride form. The ion exchanger had a bed volume of 200 L. Using a second filtration step on the cross-flow filter (150 kDa cut-off) (Microdyn-Nadir, Wiesbaden, Germany), precipitates originating from acidifying the solution were removed. For concentration of the sugar, the solution was nanofiltrated on a Dow FILMTECH NF270-4040 (Inaqua, Mönchengladbach, Germany), or, alternatively on a Trisep 4040-XN45-TSF Membrane (0.5 kDa cut-off) (Microdyn-Nadir, Wiesbaden, Germany). Using the latter, the monosaccharide *N*-acetylglucosamine, originating from the fermentation process and contaminating the sialyllactose solution, was separated from the product. The concentrated sialyllactose solution was then treated with activated charcoal (CAS:7440-44-0, Carl Roth, Karlsruhe, Germany) to remove colorants such as Maillard reaction products and aldol reaction products. In order to separate the sialyllactose from by-products that originate from the fermentation process like sialic acid and *N*-acetylglucosamine, the solution was filtrated on with a 1 kDa cut-off membrane GE4040F30 (GE water & process technologies, Ratingen, Germany), and diafiltrated to a conductivity of 0.6 to 0.8 mS cm⁻¹. The diluted solution was concentrated on a rotary evaporator to a concentration of about 300 g/L. In a final chromatographic separation other contaminating sugars, like di-sialyllactose were removed. Therefor the concentrated solution was applied to a weak anion ion exchange resin in the acetate form (Amberlite FPA51, Dow Chemical, Michigan, USA). While the sialyllactose rarely binds to the resin, the di-sialyllactose is adsorbed. Thus, the sialyllactose is eluted with 10 mM ammoniumacetat, while the di-sialyllactose is eluted with 1 M ammoniumacetat. For removal of the ammoniumacetat, the sialyllactose was precipitated with a 10-fold excess of ethanol. The solid fraction was filtrated and dried.

The product was finalized by passing a 20% sialyllactose solution sequentially through a 6 kDa filter (Pall Microza ultrafiltration module SIP-2013, Pall Corporation, Dreieich, Germany) and a 0.2 µm sterile filter.

A part of the solution was spray dried using a Büchi spray dryer (Büchi Mini Spray Dryer B-290) (Büchi, Essen, Germany), applying the following parameters: Inlet-

temperature: 130°C, Outlet temperature 67°C-71°C, gasflow 670 L/h, aspirator 100%.

The spray-dried 6'-sialyllactose had a purity of 91%, while the 3'-sialyllactose material had a purity of 93%.

5 Example 5: Preparations of HMO mixtures

Mixtures of HMOs were prepared from solid products. Therefor the single HMOs were spray- dried and the powder were mixed. HMO-Mix I contained 2'-fucosyllactose and lacto-*N*-tetraose in a ratio of 70% to 30%; HMO-Mix II contained 2'-fucosyllactose (52%), 3-fucosyllactose (13%), lacto-*N*-tetraose (26%), 3'-sialyllactose (4%), and 6'-sialyllactose (5%). The mixed powders were solved in water to a solution of 20% sugar, and spray-died again using the Büchi spray dryer as described in example 4.

Example 6: Characterisation of spray-dried human milk oligosaccharides

6.1 Differential scanning calorimetry (DSC)

Using differential scanning calorimetry (DSC) on a Mettler Toledo 821e (Mettler Toledo, Giessen, Germany) thermal events of spray-dried human milk oligosaccharides, namely 3-fucosyllactose, 6'-sialyllactose, 3'-sialyllactose, lacto-*N*-tetraose, and spray-dried mixtures of human milk oligosaccharides, a mixture (HMO-Mix I) of 2'-fucosyllactose/lacto-*N*-tetraose, and a mixture (HMO Mix II) of 2'-fucosyllactose, 3-fucosyllactose, lacto-*N*-tetraose, 6'-sialyllactose, 3'-sialyllactose, respectively, were determined.

A Mettler Toledo 821e (Mettler Toledo, Giessen, Germany) was used to determine thermal events of the spray-dried products (glass transition temperature (*T_g*), further exo- and endothermic events).

Approximately 25 mg of the spray-dried human milk oligosaccharides were analyzed in crimped Al-crucibles (Mettler Toledo, Giessen, Germany). The samples were cooled to 0 °C with 10 K/min and reheated to 100 °C with a scanning rate of

10 K/min. After cooling down the samples to 0 °C in a second heating cycle, the samples were reheated to 150 °C. The midpoint of the endothermic shift of the baseline during the heating scan was taken as glass transition temperature (T_g). Exothermic and endothermic peaks are reported by means of the peak temperature and the normalized energy of the event.

The first heating scan in all samples showed a main glass transition event in the total heat flow, as evidenced by a main step transition in the range of approximately 48-58 °C, in most of the samples, the major glass transition event observed in the first heating scan reoccurred in the second heating scan. The results of the DSC analyses are summarized in table 4.

Sample	1 st heating scan	2 nd heating scan
	T _g [°C]	T _g [°C]
3-fucosyllactose	57,6	59,9
lacto- <i>N</i> -tetraose	49,9	79,4
6'-sialyllactose	47,6	49,6
3'-sialyllactose	48,8	54,3
2'-fucosyllactose/lacto- <i>N</i> -tetraose	56,3	59
HMO Mix	54,2	55,6

Table 4: Thermal events of HMOs as determined by differential scanning calorimetry

For 3-fucosyllactose an endothermal relaxation peak after T_g in the first heating scan was detected. For lacto-*N*-tetraose a much higher T_g of about 79 °C was detected in the 2nd heating scan compared to that of the other samples. This might be caused by an endothermal event during the first heating scan at about 89 °C (-6.04 J/g). Like for 3-fucosyllactose, also for 6'-sialyllactose an endothermal relaxation peak was detected after T_g, however, in this sample additionally an endothermal event occurred at 77°C (-0.22 J/g). No endothermal events were detected for the 3'-sialyllactose and the HMO-Mix I, for HMO-Mix II the endothermal event during the 1st heating scan was at 79°C (0.34 J/g).

6.2 X-ray powder diffraction (XRD)

Wide angle X-ray powder diffraction (XRD) was used to study the morphology of lyophilized products. The X-ray diffractometer Empyrean (Panalytical, Almelo, The Netherlands) equipped with a copper anode (45 kV, 40 mA, $K_{\alpha 1}$ emission at a wavelength of 0.154 nm) and a PIXcel3D detector was used. Approximately 100 mg the spray-dried samples were analyzed in reflection mode in the angular range from 5-45° 2 θ , with a step size of 0.04° 2 θ and a counting time of 100 seconds per step.

All singular oligosaccharides as well as the HMO Mixes I and II showed a fully amorphous state (Figures 1 to 6). For lacto-*N*-tetraose a second (amorphous) signal was detected around 9-10°.

6.3 Laser diffraction

The powder particle size was assessed by laser diffraction. The system detects scattered and diffracted light by an array of concentrically arranged sensor elements. The software-algorithm is then approximating the particle counts by calculating the z-values of the light intensity values, which arrive at the different sensor elements. The analysis was executed using a SALD-7500 Aggregate Sizer (Shimadzu Corporation, Kyoto, Japan) quantitative laser diffraction system (qLD).

A small amount (spatula tip) of each sample was dispersed in 2 ml isooctane and homogenized by ultrasonication for five minutes. The dispersion was transferred into a batch cell filled with isooctane and analyzed in manual mode.

Data acquisition settings were as follows: Signal Averaging Count per Measurement: 128, Signal Accumulation Count: 3, and Interval: 2 seconds.

Prior to measurement, the system was blanked with isooctane. Each sample dispersion was measured 3 times and the mean values and the standard deviation are reported. Data was evaluated using software WING SALD II version V3.1. Since the refractive index of the sample was unknown, the refractive index of sugar (disaccharide) particles (1.530) was used for determination of size distribution profiles. Size values for mean and median diameter are reported.

The mean particle sizes for all samples were very similar, slightly lower values were measured for HMO-Mix II. The particle size characteristics are summarized in Table 5. In addition, the particle size distribution showed the presence of one main size population for all of the samples.

5

Size	3-fucosyl-lactose	lacto- <i>N</i> -tetraose	6'-sialyllactose	3'-sialyllactose	HMO-Mix I	HMO Mix II
Mean [nm]	119.2 ± 0.5	117.3 ± 0.7	113.8 ± 1.5	115.4 ± 0.6	113.1 ± 0.3	97.3 ± 5.3
Median [nm]	141.3 ± 0.0	141.3 ± 0.0	141.3 ± 0.0	121.9 ± 16.7	141.3 ± 0.0	112.2 ± 0.0

Table 5: Particle size of HMOs as determined by laser diffraction