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(54) Title: PRODUCTION OF A MIXTURE OF MAMMALIAN MILK OLIGOSACCHARIDES BY A CELL

(57) Abstract: The present invention is in the technical field of synthetic biology and metabolic engineering. More particularly, the present invention is in the technical field of cultivation or fermentation of metabolically engineered cells. The present invention describes a cell metabolically engineered for production of a mixture of at least three different mammalian milk oligosaccharides. Furthermore, the present invention provides a method for the production of a mixture of at least three different mammalian milk oligosaccharides by a cell as well as the purification of at least one of said mammalian milk oligosaccharides from the cultivation.

Production of a mixture of mammalian milk oligosaccharides by a cell

The present invention is in the technical field of synthetic biology and metabolic engineering. More particularly, the present invention is in the technical field of cultivation or fermentation of metabolically engineered cells. The present invention describes a cell metabolically engineered for production of a mixture of at least three different mammalian milk oligosaccharides. Furthermore, the present invention provides a method for the production of a mixture of at least three different mammalian milk oligosaccharides by a cell as well as the purification of at least one of said mammalian milk oligosaccharides from the cultivation.

Background

Mammalian milk oligosaccharides (MMOs) are a group of complex sugars present in mammal's milk. The oligosaccharide fraction is a major component in mammalian milk. MMOs are involved in many vital phenomena such as differentiation, development, biological recognition processes and immunological processes and can function e.g. as prebiotics, antiadhesives, and antimicrobials. This variety in functionality might explain the great diversity of their structures (Boehm and Stahl, J. Nutrition 137(3) (2007) 847S-849S; Bode L., Early Human Devel. 91(11) (2015) 619-622). There is large scientific and commercial interest in mixtures of MMOs due to the wide functional spectrum of this type of oligosaccharides. Yet, the availability of mixtures of MMOs is limited as production relies on chemical or chemo-enzymatic synthesis or on purification from natural sources. Chemical synthesis methods are laborious and time-consuming and because of the large number of steps involved they are difficult to scale-up. Enzymatic approaches using glycosyltransferases offer many advantages above chemical synthesis. Glycosyltransferases catalyze the transfer of a sugar moiety from an activated nucleotide-sugar donor onto saccharide or non-saccharide acceptors (Coutinho et al., J. Mol. Biol. 328 (2003) 307-317). These glycosyltransferases are the source for biotechnologists to synthesize MMOs and are used both in (chemo)enzymatic approaches as well as in cell-based production systems. However, stereospecificity and regioselectivity of glycosyltransferases are still a formidable challenge. In addition, chemo-enzymatic approaches need to regenerate in situ nucleotide-sugar donors. Cellular production of MMOs needs tight control of spatiotemporal availability of adequate levels of nucleotide-sugar donors in proximity of complementary glycosyltransferases. Due to these difficulties, current methods often result in the synthesis of a single MMO instead of a mixture of MMOs.

It is an object of the present invention to provide for tools and methods by means of which a mammalian milk oligosaccharide mixture comprising at least three different MMOs can be produced by a cell, preferably a single cell, in an efficient, time and cost-effective way and if needed, continuous process.

According to the invention, this and other objects are achieved by providing a cell and a method for the production of a mammalian milk oligosaccharide mixture comprising at least three different MMOs

wherein the cell is genetically modified for the production of said MMOs.

Description

Surprisingly, it has now been found that it is possible to produce mixtures of MMOs comprising at least three different MMOs by a single cell. The present invention provides a metabolically engineered cell and a method for the production of a mammalian milk oligosaccharide mixture comprising at least three different MMOs. The method comprises the steps of providing a cell which expresses at least two glycosyltransferases and is capable to synthesize (a) nucleotide-sugar(s) that is/are donor(s) for said glycosyltransferases, and cultivating said cell under conditions permissive for producing said mixture of MMOs. The present invention also provides methods to separate at least one, preferably all, of said MMOs from the mammalian milk oligosaccharide mixture. Furthermore, the present invention provides a cell metabolically engineered for production of a mixture of MMOs comprising at least three different MMOs.

Definitions

The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus, if an element can be understood in the context of this specification as including more than one meaning, then its use in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself.

The various embodiments and aspects of embodiments of the invention disclosed herein are to be understood not only in the order and context specifically described in this specification, but to include any order and any combination thereof. Whenever the context requires, all words used in the singular number shall be deemed to include the plural and vice versa. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described herein are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, purification steps are performed according to the manufacturer's specifications.

In the specification, there have been disclosed embodiments of the invention, and although specific terms are employed, the terms are used in a descriptive sense only and not for purposes of limitation, the scope of the invention being set forth in the following claims. It must be understood that the illustrated embodiments have been set forth only for the purposes of example and that it should not be taken as limiting the invention. It will be apparent to those skilled in the art that alterations, other embodiments, improvements, details and uses can be made consistent with the letter and spirit of the invention herein and within the scope of this invention, which is limited only by the claims, construed in accordance with

the patent law, including the doctrine of equivalents. In the claims which follow, reference characters used to designate claim steps are provided for convenience of description only, and are not intended to imply any particular order for performing the steps.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. Throughout the application, the verb "to comprise" may be replaced by "to consist" or "to consist essentially of" and vice versa. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a composition as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". Throughout the application, unless explicitly stated otherwise, the articles "a" and "an" are preferably replaced by "at least two", more preferably by "at least three", even more preferably by "at least four", even more preferably by "at least five", even more preferably by "at least six", most preferably by "at least seven".

Each embodiment as identified herein may be combined together unless otherwise indicated. All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. The full content of the priority applications, including EP20190198, EP20190200, EP20190204 and EP20190205, are also incorporated by reference to the same extent as if said priority applications were specifically and individually indicated to be incorporated by reference.

According to the present invention, the term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are

"polynucleotide(s)" according to the present invention. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, are to be understood to be covered by the term "polynucleotides". It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. The term "polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to the skilled person. The same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Furthermore, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid sidechains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

The term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide of the invention. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by integrated phage or an insertion sequence or editing) together with additional regions that also may contain coding and/or non-coding sequences.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been

changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Similarly, a "synthetic" sequence, as the term is used herein, means any sequence that has been generated synthetically and not directly isolated from a natural source. "Synthesized", as the term is used herein, means any synthetically generated sequence and not directly isolated from a natural source.

The terms "recombinant" or "transgenic" or "metabolically engineered" or "genetically modified", as used herein with reference to a cell or host cell are used interchangeably and indicates that the cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid (i.e., a sequence "foreign to said cell" or a sequence "foreign to said location or environment in said cell"). Such cells are described to be transformed with at least one heterologous or exogenous gene, or are described to be transformed by the introduction of at least one heterologous or exogenous gene. Metabolically engineered or recombinant or transgenic cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The terms also encompass cells that contain a nucleic acid endogenous to the cell that has been modified or its expression or activity has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, replacement of a promoter; site-specific mutation; and related techniques. Accordingly, a "recombinant polypeptide" is one which has been produced by a recombinant cell. A "heterologous sequence" or a "heterologous nucleic acid", as used herein, is one that originates from a source foreign to the particular cell (e.g. from a different species), or, if from the same source, is modified from its original form or place in the genome. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was derived, or, if from the same source, is modified from its original form or place in the genome. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microorganism cell, wherein techniques may be applied which will depend on the cell and the sequence that is to be introduced. Various techniques are known to a person skilled in the art and are, e.g., disclosed in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). The term "mutant" cell or microorganism as used within the context of the present invention refers to a cell or microorganism which is genetically modified.

The term "endogenous," within the context of the present invention refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell and is occurring at its natural location in the cell chromosome and of which the control of expression has not been altered compared to the natural control mechanism acting on its expression. The term "exogenous" refers to any polynucleotide,

polypeptide or protein sequence which originates from outside the cell under study and not a natural part of the cell or which is not occurring at its natural location in the cell chromosome or plasmid.

The term "heterologous" when used in reference to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme refers to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is from a source or derived from a source other than the host organism species. In contrast a "homologous" polynucleotide, gene, nucleic acid, polypeptide, or enzyme is used herein to denote a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is derived from the host organism species. When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (e.g. a promoter, a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, etc.), "heterologous" means that the regulatory sequence or auxiliary sequence is not naturally associated with the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome, or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (i.e. in the genome of a non-genetically engineered organism) is referred to herein as a "heterologous promoter," even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

The term "modified activity" of a protein or an enzyme relates to a change in activity of the protein or the enzyme compared to the wild type, i.e. natural, activity of said protein or enzyme. Said modified activity can either be an abolished, impaired, reduced or delayed activity of said protein or enzyme compared to the wild type activity of the protein or the enzyme but can also be an accelerated or an enhanced activity of said protein or the enzyme compared to the wild type activity of the protein or the enzyme. A modified activity of a protein or an enzyme is obtained by modified expression of said protein or enzyme or is obtained by expression of a modified, i.e. mutant form of the protein or enzyme. A modified activity of an enzyme further relates to a modification in the apparent Michaelis constant K_m and/or the apparent maximal velocity (V_{max}) of the enzyme.

The term "modified expression" of a gene relates to a change in expression compared to the wild type expression of said gene in any phase of the production process of the encoded protein. Said modified expression is either a lower or higher expression compared to the wild type, wherein the term "higher expression" is also defined as "overexpression" of said gene in the case of an endogenous gene or "expression" in the case of a heterologous gene that is not present in the wild type strain. Lower expression or reduced expression is obtained by means of common well-known technologies for a skilled person (such as the usage of siRNA, CrispR, CrispRi, riboswitches, recombineering, homologous recombination, ssDNA mutagenesis, RNAi, miRNA, asRNA, mutating genes, knocking-out genes, transposon mutagenesis, ...) which are used to change the genes in such a way that they are less-able (i.e. statistically significantly 'less-able' compared to a functional wild-type gene) or completely unable (such

as knocked-out genes) to produce functional final products. The term “riboswitch” as used herein is defined to be part of the messenger RNA that folds into intricate structures that block expression by interfering with translation. Binding of an effector molecule induces conformational change(s) permitting regulated expression post-transcriptionally. Next to changing the gene of interest in such a way that lower expression is obtained as described above, lower expression can also be obtained by changing the transcription unit, the promoter, an untranslated region, the ribosome binding site, the Shine Dalgarno sequence or the transcription terminator. Lower expression or reduced expression can for instance be obtained by mutating one or more base pairs in the promoter sequence or changing the promoter sequence fully to a constitutive promoter with a lower expression strength compared to the wild type or an inducible promoter which result in regulated expression or a repressible promoter which results in regulated expression. Overexpression or expression is obtained by means of common well-known technologies for a skilled person (such as the usage of artificial transcription factors, de novo design of a promoter sequence, ribosome engineering, introduction or re-introduction of an expression module at euchromatin, usage of high-copy-number plasmids), wherein said gene is part of an “expression cassette” which relates to any sequence in which a promoter sequence, untranslated region sequence (containing either a ribosome binding sequence, Shine Dalgarno or Kozak sequence), a coding sequence and optionally a transcription terminator is present, and leading to the expression of a functional active protein. Said expression is either constitutive or regulated.

The term “constitutive expression” is defined as expression that is not regulated by transcription factors other than the subunits of RNA polymerase (e.g. the bacterial sigma factors like σ^{70} , σ^{54} , or related σ -factors and the yeast mitochondrial RNA polymerase specificity factor MTF1 that co-associate with the RNA polymerase core enzyme) under certain growth conditions. Non-limiting examples of such transcription factors are CRP, LacI, ArcA, Cra, IclR in *E. coli*, or, Aft2p, Crz1p, Skn7 in *Saccharomyces cerevisiae*, or, DeoR, GntR, Fur in *B. subtilis*. These transcription factors bind on a specific sequence and may block or enhance expression in certain growth conditions. The RNA polymerase is the catalytic machinery for the synthesis of RNA from a DNA template. RNA polymerase binds a specific sequence to initiate transcription, for instance via a sigma factor in prokaryotic hosts or via MTF1 in yeasts. Constitutive expression offers a constant level of expression with no need for induction or repression.

The term “expression by a natural inducer” is defined as a facultative or regulatory expression of a gene that is only expressed upon a certain natural condition of the host (e.g. organism being in labor, or during lactation), as a response to an environmental change (e.g. including but not limited to hormone, heat, cold, light, oxidative or osmotic stress / signaling), or dependent on the position of the developmental stage or the cell cycle of said host cell including but not limited to apoptosis and autophagy.

The term “control sequences” refers to sequences recognized by the cells transcriptional and translational systems, allowing transcription and translation of a polynucleotide sequence to a polypeptide. Such DNA sequences are thus necessary for the expression of an operably linked coding sequence in a particular cell

or organism. Such control sequences can be, but are not limited to, promoter sequences, ribosome binding sequences, Shine Dalgarno sequences, Kozak sequences, transcription terminator sequences. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. DNA for a presequence or secretory leader may be operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Said control sequences can furthermore be controlled with external chemicals, such as, but not limited to, IPTG, arabinose, lactose, allo-lactose, rhamnose or fucose via an inducible promoter or via a genetic circuit that either induces or represses the transcription or translation of said polynucleotide to a polypeptide.

Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. The term "wild type" refers to the commonly known genetic or phenotypical situation as it occurs in nature.

The term "modified expression of a protein" as used herein refers to i) higher expression or overexpression of an endogenous protein, ii) expression of a heterologous protein or iii) expression and/or overexpression of a variant protein that has a higher activity compared to the wild-type (i.e. native) protein.

As used herein, the term "mammary cell(s)" generally refers to mammary epithelial cell(s), mammary-epithelial luminal cell(s), or mammalian epithelial alveolar cell(s), or any combination thereof. As used herein, the term "mammary-like cell(s)" generally refers to cell(s) having a phenotype/genotype similar (or substantially similar) to natural mammary cell(s) but is/are derived from non-mammary cell source(s). Such mammary-like cell(s) may be engineered to remove at least one undesired genetic component and/or to include at least one predetermined genetic construct that is typical of a mammary cell. Non-limiting examples of mammary-like cell(s) may include mammary epithelial-like cell(s), mammary epithelial luminal-like cell(s), non-mammary cell(s) that exhibits one or more characteristics of a cell of a mammary cell lineage, or any combination thereof. Further non-limiting examples of mammary-like cell(s) may include cell(s) having a phenotype similar (or substantially similar) to natural mammary cell(s), or more particularly a phenotype similar (or substantially similar) to natural mammary epithelial cell(s). A cell with a phenotype or that exhibits at least one characteristic similar to (or substantially similar to) a natural mammary cell or a mammary epithelial cell may comprise a cell (e.g., derived from a mammary cell lineage or a non-mammary cell lineage) that exhibits either naturally, or has been engineered to, be capable of expressing at least one milk component.

As used herein, the term “non-mammary cell(s)” may generally include any cell of non-mammary lineage. In the context of the invention, a non-mammary cell can be any mammalian cell capable of being engineered to express at least one milk component. Non-limiting examples of such non-mammary cell(s) include hepatocyte(s), blood cell(s), kidney cell(s), cord blood cell(s), epithelial cell(s), epidermal cell(s), myocyte(s), fibroblast(s), mesenchymal cell(s), or any combination thereof. In some instances, molecular biology and genome editing techniques can be engineered to eliminate, silence, or attenuate myriad genes simultaneously.

Throughout the application, unless explicitly stated otherwise, the expressions “capable of...<verb>” and “capable to...<verb>” are preferably replaced with the active voice of said verb and *vice versa*. For example, the expression “capable of expressing” is preferably replaced with “expresses” and *vice versa*, i.e. “expresses” is preferably replaced with “capable of expressing”.

“Variant(s)” as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to the persons skilled in the art.

The term “derivative” of a polypeptide, as used herein, is a polypeptide which may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence of the polypeptide, but which result in a silent change, thus producing a functionally equivalent polypeptide. Amino acid substitutions may be made based on similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; planar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Within the context of this invention, a derivative polypeptide as used herein, refers to a polypeptide capable of exhibiting a

substantially similar in vitro and/or in vivo activity as the original polypeptide as judged by any of a number of criteria, including but not limited to enzymatic activity, and which may be differentially modified during or after translation. Furthermore, non-classical amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the original polypeptide sequence.

5 In some embodiments, the present invention contemplates making functional variants by modifying the structure of an enzyme as used in the present invention. Variants can be produced by amino acid substitution, deletion, addition, or combinations thereof. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g.,
10 conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of a polypeptide of the invention results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type polypeptide.

15 The term "functional homolog" as used herein describes those molecules that have sequence similarity (in other words, homology) and also share at least one functional characteristic such as a biochemical activity (Altenhoff *et al.*, PLoS Comput. Biol. 8 (2012) e1002514). Functional homologs will typically give rise to the same characteristics to a similar, but not necessarily the same, degree. Functionally homologous proteins give the same characteristics where the quantitative measurement produced by
20 one homolog is at least 10 percent of the other; more typically, at least 20 percent, between about 30 percent and about 40 percent; for example, between about 50 percent and about 60 percent; between about 70 percent and about 80 percent; or between about 90 percent and about 95 percent; between about 98 percent and about 100 percent, or greater than 100 percent of that produced by the original molecule. Thus, where the molecule has enzymatic activity the functional homolog will have the above-
25 recited percent enzymatic activities compared to the original enzyme. Where the molecule is a DNA-binding molecule (e.g., a polypeptide) the homolog will have the above-recited percentage of binding affinity as measured by weight of bound molecule compared to the original molecule.

A functional homolog and the reference polypeptide may be naturally occurring polypeptides, and the sequence similarity may be due to convergent or divergent evolutionary events. Functional homologs are
30 sometimes referred to as orthologs, where "ortholog", refers to a homologous gene or protein that is the functional equivalent of the referenced gene or protein in another species.

Orthologous genes are homologous genes in different species that originate by vertical descent from a single gene of the last common ancestor, wherein the gene and its main function are conserved. A homologous gene is a gene inherited in two species by a common ancestor.

35 The term "ortholog" when used in reference to an amino acid or nucleotide/nucleic acid sequence from a given species refers to the same amino acid or nucleotide/nucleic acid sequence from a different species.

It should be understood that two sequences are orthologs of each other when they are derived from a common ancestor sequence via linear descent and/or are otherwise closely related in terms of both their sequence and their biological function. Orthologs will usually have a high degree of sequence identity but may not (and often will not) share 100% sequence identity.

- 5 Paralogous genes are homologous genes that originate by a gene duplication event. Paralogous genes often belong to the same species, but this is not necessary. Paralogs can be split into in-paralogs (paralogous pairs that arose after a speciation event) and out-paralogs (paralogous pairs that arose before a speciation event). Between species out-paralogs are pairs of paralogs that exist between two organisms due to duplication before speciation. Within species out-paralogs are pairs of paralogs that exist in the
10 same organism, but whose duplication event happened after speciation. Paralogs typically have the same or similar function.

Functional homologs can be identified by analysis of nucleotide and polypeptide sequence alignments. For example, performing a query on a database of nucleotide or polypeptide sequences can identify homologs of the polypeptide of interest like e.g. a biomass-modulating polypeptide, a glycosyltransferase,
15 a protein involved in nucleotide-activated sugar synthesis or a membrane protein. Sequence analysis can involve BLAST, Reciprocal BLAST, or PSI-BLAST analysis of non-redundant databases using amino acid sequence of a biomass-modulating polypeptide, a glycosyltransferase, a protein involved in nucleotide-activated sugar synthesis or a membrane protein, respectively, as the reference sequence. Amino acid sequence is, in some instances, deduced from the nucleotide sequence. Typically, those polypeptides in
20 the database that have greater than 40 percent sequence identity are candidates for further evaluation for suitability as a biomass-modulating polypeptide, a glycosyltransferase, a protein involved in nucleotide-activated sugar synthesis or a membrane protein, respectively. Amino acid sequence similarity allows for conservative amino acid substitutions, such as substitution of one hydrophobic residue for another or substitution of one polar residue for another or substitution of one acidic amino acid for
25 another or substitution of one basic amino acid for another etc. Preferably, by conservative substitutions is intended combinations such as glycine by alanine and *vice versa*; valine, isoleucine and leucine by methionine and *vice versa*; aspartate by glutamate and *vice versa*; asparagine by glutamine and *vice versa*; serine by threonine and *vice versa*; lysine by arginine and *vice versa*; cysteine by methionine and *vice versa*; and phenylalanine and tyrosine by tryptophan and *vice versa*. If desired, manual inspection of such
30 candidates can be carried out in order to narrow the number of candidates to be further evaluated. Manual inspection can be performed by selecting those candidates that appear to have domains present in productivity-modulating polypeptides, e.g., conserved functional domains.

"Fragment", with respect to a polynucleotide, refers to a clone or any part of a polynucleotide molecule, particularly a part of a polynucleotide that retains a usable, functional characteristic of the full-length
35 polynucleotide molecule. Useful fragments include oligonucleotides and polynucleotides that may be used in hybridization or amplification technologies or in the regulation of replication, transcription or

translation. A "polynucleotide fragment" refers to any subsequence of a polynucleotide SEQ ID NO (or Genbank NO.), typically, comprising or consisting of at least about 9, 10, 11, 12 consecutive nucleotides, for example at least about 30 nucleotides or at least about 50 nucleotides of any of the polynucleotide sequences provided herein. Exemplary fragments can additionally or alternatively include fragments that

5 comprise, consist essentially of, or consist of a region that encodes a conserved family domain of a polypeptide. Exemplary fragments can additionally or alternatively include fragments that comprise a conserved domain of a polypeptide. As such, a fragment of a polynucleotide SEQ ID NO (or Genbank NO.) preferably means a nucleotide sequence which comprises or consists of said polynucleotide SEQ ID NO (or Genbank NO.) wherein no more than 200, 150, 100, 50 or 25 consecutive nucleotides are missing,

10 preferably no more than 50 consecutive nucleotides are missing, and which retains a usable, functional characteristic (e.g. activity) of the full-length polynucleotide molecule which can be assessed by the skilled person through routine experimentation. Alternatively, a fragment of a polynucleotide SEQ ID NO (or Genbank NO.) preferably means a nucleotide sequence which comprises or consists of an amount of consecutive nucleotides from said polynucleotide SEQ ID NO (or Genbank NO.) and wherein said amount

15 of consecutive nucleotides is at least 50.0 %, 60.0 %, 70.0 %, 80.0 %, 81.0 %, 82.0 %, 83.0 %, 84.0 %, 85.0 %, 86.0 %, 87.0 %, 88.0 %, 89.0 %, 90.0 %, 91.0 %, 92.0 %, 93.0 %, 94.0 %, 95.0 %, 95.5 %, 96.0 %, 96.5 %, 97.0 %, 97.5 %, 98.0 %, 98.5 %, 99.0 %, 99.5 %, 100 %, preferably at least 80%, more preferably at least 87%, even more preferably at least 90%, even more preferably at least 95%, most preferably at least 97%, of the full-length of said polynucleotide SEQ ID NO (or Genbank NO.) and retains a usable, functional

20 characteristic (e.g. activity) of the full-length polynucleotide molecule. As such, a fragment of a polynucleotide SEQ ID NO (or Genbank NO.) preferably means a nucleotide sequence which comprises or consists of said polynucleotide SEQ ID NO (or Genbank NO.), wherein an amount of consecutive nucleotides is missing and wherein said amount is no more than 50.0 %, 40.0 %, 30.0 % of the full-length of said polynucleotide SEQ ID NO (or Genbank NO.), preferably no more than 20.0 %, 15.0 %, 10.0 %, 9.0

25 %, 8.0 %, 7.0 %, 6.0 %, 5.0 %, 4.5 %, 4.0 %, 3.5 %, 3.0 %, 2.5 %, 2.0 %, 1.5 %, 1.0 %, 0.5 %, more preferably no more than 15%, even more preferably no more than 10%, even more preferably no more than 5%, most preferably no more than 2.5%, of the full-length of said polynucleotide SEQ ID NO (or Genbank NO.) and wherein said fragment retains a usable, functional characteristic (e.g. activity) of the full-length polynucleotide molecule which can be routinely assessed by the skilled person.

30 Throughout the application, the sequence of a polynucleotide can be represented by a SEQ ID NO or alternatively by a GenBank NO. Therefore, the terms "polynucleotide SEQ ID NO" and "polynucleotide GenBank NO." can be interchangeably used, unless explicitly stated otherwise.

Fragments may additionally or alternatively include subsequences of polypeptides and protein molecules, or a subsequence of the polypeptide. In some cases, the fragment or domain is a subsequence of the

35 polypeptide which performs at least one biological function of the intact polypeptide in substantially the same manner, preferably to a similar extent, as does the intact polypeptide. A "subsequence of the

polypeptide” as defined herein refers to a sequence of contiguous amino acid residues derived from the polypeptide. For example, a polypeptide fragment can comprise a recognizable structural motif or functional domain such as a DNA-binding site or domain that binds to a DNA promoter region, an activation domain, or a domain for protein-protein interactions, and may initiate transcription. Fragments can vary in size from as few as 3 amino acid residues to the full length of the intact polypeptide, for example at least about 20 amino acid residues in length, for example at least about 30 amino acid residues in length. As such, a fragment of a polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) preferably means a polypeptide sequence which comprises or consists of said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) wherein no more than 80, 60, 50, 40, 30, 20 or 15 consecutive amino acid residues are missing, preferably no more than 40 consecutive amino acid residues are missing, and performs at least one biological function of the intact polypeptide in substantially the same manner, preferably to a similar or greater extent, as does the intact polypeptide which can be routinely assessed by the skilled person. Alternatively, a fragment of a polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) preferably means a polypeptide sequence which comprises or consists of an amount of consecutive amino acid residues from said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) and wherein said amount of consecutive amino acid residues is at least 50.0 %, 60.0 %, 70.0 %, 80.0 %, 81.0 %, 82.0 %, 83.0 %, 84.0 %, 85.0 %, 86.0 %, 87.0 %, 88.0 %, 89.0 %, 90.0 %, 91.0 %, 92.0 %, 93.0 %, 94.0 %, 95.0 %, 95.5 %, 96.0 %, 96.5 %, 97.0 %, 97.5 %, 98.0 %, 98.5 %, 99.0 %, 99.5 %, 100 %, preferably at least 80%, more preferably at least 87%, even more preferably at least 90%, even more preferably at least 95%, most preferably at least 97% of the full-length of said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) and which performs at least one biological function of the intact polypeptide in substantially the same manner, preferably to a similar or greater extent, as does the intact polypeptide which can be routinely assessed by the skilled person. As such, a fragment of a polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) preferably means a polypeptide sequence which comprises or consists of said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.), wherein an amount of consecutive amino acid residues is missing and wherein said amount is no more than 50.0 %, 40.0 %, 30.0 % of the full-length of said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.), preferably no more than 20.0 %, 15.0 %, 10.0 %, 9.0 %, 8.0 %, 7.0 %, 6.0 %, 5.0 %, 4.5 %, 4.0 %, 3.5 %, 3.0 %, 2.5 %, 2.0 %, 1.5 %, 1.0 %, 0.5 %, more preferably no more than 15%, even more preferably no more than 10%, even more preferably no more than 5%, most preferably no more than 2.5%, of the full-length of said polypeptide SEQ ID NO (or UniProt ID or Genbank NO.) and which performs at least one biological function of the intact polypeptide in substantially the same manner, preferably to a similar or greater extent, as does the intact polypeptide which can be routinely assessed by the skilled person.

Throughout the application, the sequence of a polypeptide can be represented by a SEQ ID NO or alternatively by an UniProt ID or GenBank NO. Therefore, the terms “polypeptide SEQ ID NO” and “polypeptide UniProt ID” and “polypeptide GenBank NO.” can be interchangeably used, unless explicitly

stated otherwise.

Preferentially, a fragment of a polypeptide is a functional fragment that has at least one property or activity of the polypeptide from which it is derived, preferably to a similar or greater extent. A functional fragment can for example, include a functional domain or conserved domain of a polypeptide. It is understood that a polypeptide or a fragment thereof may have conservative amino acid substitutions which have substantially no effect on the polypeptide's activity. By conservative substitutions is intended substitutions of one hydrophobic amino acid for another or substitution of one polar amino acid for another or substitution of one acidic amino acid for another or substitution of one basic amino acid for another etc. Preferably, by conservative substitutions is intended combinations such as glycine by alanine and *vice versa*; valine, isoleucine and leucine by methionine and *vice versa*; aspartate by glutamate and *vice versa*; asparagine by glutamine and *vice versa*; serine by threonine and *vice versa*; lysine by arginine and *vice versa*; cysteine by methionine and *vice versa*; and phenylalanine and tyrosine by tryptophan and *vice versa*. A domain can be characterized, for example, by a Pfam (El-Gebali et al., Nucleic Acids Res. 47 (2019) D427-D432) or Conserved Domain Database (CDD) (<https://www.ncbi.nlm.nih.gov/cdd>) (Lu et al., Nucleic Acids Res. 48 (2020) D265-D268) designation. The content of each database is fixed at each release and is not to be changed. When the content of a specific database is changed, this specific database receives a new release version with a new release date. All release versions for each database with their corresponding release dates and specific content as annotated at these specific release dates are available and known to those skilled in the art. The PFAM database (<https://pfam.xfam.org/>) used herein was Pfam version 33.1 released on June 11, 2020. Protein sequence information and functional information can be provided by a comprehensive resource for protein sequence and annotation data like e.g. the Universal Protein Resource (UniProt) (www.uniprot.org) (Nucleic Acids Res. 2021, 49(D1), D480-D489). UniProt comprises the expertly and richly curated protein database called the UniProt Knowledgebase (UniProtKB), together with the UniProt Reference Clusters (UniRef) and the UniProt Archive (UniParc). The UniProt identifiers (UniProt ID) are unique for each protein present in the database. UniProt IDs as used herein are the UniProt IDs in the UniProt database version of 05 May 2021. Proteins that do not have an UniProt ID are referred herein using the respective GenBank Accession number (GenBank No.) as present in the NIH genetic sequence database (<https://www.ncbi.nlm.nih.gov/genbank/>) (Nucleic Acids Res. 2013, 41(D1), D36-D42) version of 05 May 2021.

The term "glycosyltransferase" as used herein refers to an enzyme capable to catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The as such synthesized mammalian milk oligosaccharides can be of the linear type or of the branched type and can contain multiple monosaccharide building blocks. A classification of glycosyltransferases using nucleotide diphospho-sugar, nucleotide monophospho-sugar and sugar phosphates and related proteins into distinct sequence-based families has been described (Campbell et al., Biochem. J. 326, 929-939 (1997)) and is available on the CAZy (CArbohydrate-Active EnZymes) website (www.cazy.org).

As used herein the glycosyltransferase can be selected from the list comprising but not limited to: fucosyltransferases (e.g. alpha-1,2-fucosyltransferases, alpha-1,3/1,4-fucosyltransferases, alpha-1,6-fucosyltransferases), sialyltransferases (e.g. alpha-2,3-sialyltransferases, alpha-2,6-sialyltransferases, alpha-2,8-sialyltransferases), galactosyltransferases (e.g. beta-1,3-galactosyltransferases, beta-1,4-galactosyltransferases, alpha-1,3-galactosyltransferases, alpha-1,4-galactosyltransferases), N-acetylglucosaminyltransferases (e.g. beta-1,3-N-acetylglucosaminyltransferases, beta-1,6-N-acetylglucosaminyltransferases), N-acetylgalactosaminyltransferases (e.g. alpha-1,3-N-acetylgalactosaminyltransferases, beta-1,3-N-acetylgalactosaminyltransferases), glucosyltransferases, mannosyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyl transferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases.

Fucosyltransferases are glycosyltransferases that transfer a fucose residue (Fuc) from a GDP-fucose (GDP-Fuc) donor onto a glycan acceptor. Fucosyltransferases comprise alpha-1,2-fucosyltransferases, alpha-1,3-fucosyltransferases, alpha-1,4-fucosyltransferases and alpha-1,6-fucosyltransferases that catalyze the transfer of a Fuc residue from GDP-Fuc onto a glycan acceptor via alpha-glycosidic bonds. Fucosyltransferases can be found but are not limited to the GT10, GT11, GT23, GT65 and GT68 CAZy families. Sialyltransferases are glycosyltransferases that transfer a sialyl group (like Neu5Ac or Neu5Gc) from a donor (like CMP-Neu5Ac or CMP-Neu5Gc) onto a glycan acceptor. Sialyltransferases comprise alpha-2,3-sialyltransferases, alpha-2,6-sialyltransferases and alpha-2,8-sialyltransferases that catalyze the transfer of a sialyl group onto a glycan acceptor via alpha-glycosidic bonds. Sialyltransferases can be found but are not limited to the GT29, GT42, GT80 and GT97 CAZy families. Galactosyltransferases are glycosyltransferases that transfer a galactosyl group (Gal) from an UDP-galactose (UDP-Gal) donor onto a glycan acceptor. Galactosyltransferases comprise beta-1,3-galactosyltransferases, beta-1,4-galactosyltransferases, alpha-1,3-galactosyltransferases and alpha-1,4-galactosyltransferases that transfer a Gal residue from UDP-Gal onto a glycan acceptor via alpha- or beta-glycosidic bonds. Galactosyltransferases can be found but are not limited to the GT2, GT6, GT8, GT25 and GT92 CAZy families. Glucosyltransferases are glycosyltransferases that transfer a glucosyl group (Glc) from an UDP-glucose (UDP-Glc) donor onto a glycan acceptor. Glucosyltransferases comprise alpha-glucosyltransferases, beta-1,2-glucosyltransferases, beta-1,3-glucosyltransferases and beta-1,4-glucosyltransferases that transfer a Glc residue from UDP-Glc onto a glycan acceptor via alpha- or beta-glycosidic bonds. Glucosyltransferases can be found but are not limited to the GT1, GT4 and GT25 CAZy families. Mannosyltransferases are glycosyltransferases that transfer a mannose group (Man) from a GDP-mannose (GDP-Man) donor onto a glycan acceptor. Mannosyltransferases comprise alpha-1,2-mannosyltransferases, alpha-1,3-mannosyltransferases and alpha-1,6-mannosyltransferases that transfer

a Man residue from GDP-Man onto a glycan acceptor via alpha-glycosidic bonds. Mannosyltransferases can be found but are not limited to the GT22, GT39, GT62 and GT69 CAZy families. N-acetylglucosaminyltransferases are glycosyltransferases that transfer an N-acetylglucosamine group (GlcNAc) from an UDP-N-acetylglucosamine (UDP-GlcNAc) donor onto a glycan acceptor. N-acetylglucosaminyltransferases can be found but are not limited to GT2 and GT4 CAZy families.

N-acetylgalactosaminyltransferases are glycosyltransferases that transfer an N-acetylgalactosamine group (GalNAc) from an UDP-N-acetylgalactosamine (UDP-GalNAc) donor onto a glycan acceptor. N-acetylgalactosaminyltransferases can be found but are not limited to GT7, GT12 and GT27 CAZy families.

N-acetylmannosaminyltransferases are glycosyltransferases that transfer an N-acetylmannosamine group (ManNAc) from an UDP-N-acetylmannosamine (UDP-ManNAc) donor onto a glycan acceptor.

Xylosyltransferases are glycosyltransferases that transfer a xylose residue (Xyl) from an UDP-xylose (UDP-Xyl) donor onto a glycan acceptor. Xylosyltransferases can be found but are not limited to GT61 and GT77 CAZy families. Glucuronyltransferases are glycosyltransferases that transfer a glucuronate from an UDP-glucuronate donor onto a glycan acceptor via alpha- or beta-glycosidic bonds. Glucuronyltransferases can be found but are not limited to GT4, GT43 and GT93 CAZy families.

Galacturonyltransferases are glycosyltransferases that transfer a galacturonate from an UDP-galacturonate donor onto a glycan acceptor. N-glycolylneuraminyltransferases are glycosyltransferases that transfer an N-glycolylneuraminic acid group (Neu5Gc) from a CMP-Neu5Gc donor onto a glycan acceptor. Rhamnosyltransferases are glycosyltransferases that transfer a rhamnose residue from a GDP-rhamnose donor onto a glycan acceptor. Rhamnosyltransferases can be found but are not limited to the GT1, GT2 and GT102 CAZy families. N-acetylramnosyltransferases are glycosyltransferases that transfer an N-acetylramnosamine residue from an UDP-N-acetyl-L-rhamnosamine donor onto a glycan acceptor. UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases are glycosyltransferases that use an UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose in the biosynthesis of pseudaminic acid, which is a sialic acid-like sugar that is used to modify flagellin. UDP-N-acetylglucosamine enolpyruvyl transferases (murA) are glycosyltransferases that transfer an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-N-acetylglucosamine (UDPAG) to form UDP-N-acetylglucosamine enolpyruvate. Fucosaminyltransferases are glycosyltransferases that transfer an N-acetylfucosamine residue from a dTDP-N-acetylfucosamine or an UDP-N-acetylfucosamine donor onto a glycan acceptor.

The terms "nucleotide-sugar", "nucleotide-activated sugar", or "activated sugar" are used herein interchangeably and refer to activated forms of monosaccharides. Examples of activated monosaccharides include but are not limited to UDP-galactose (UDP-Gal), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-fucose (GDP-Fuc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylfucosamine,

UDP-N-acetylfucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-acetylneuraminic acid (CMP-Neu5Ac), CMP-Neu4Ac, CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, or UDP-xylose. Nucleotide-sugars act as glycosyl donors in glycosylation reactions. Glycosylation reactions are reactions that are catalyzed by glycosyltransferases.

"Oligosaccharide" as the term is used herein and as generally understood in the state of the art, refers to a saccharide polymer containing a small number, typically three to twenty, of simple sugars, i.e. monosaccharides. The monosaccharides as used herein are reducing sugars. The oligosaccharides can be reducing or non-reducing sugars and have a reducing and a non-reducing end. A reducing sugar is any sugar that is capable of reducing another compound and is oxidized itself, that is, the carbonyl carbon of the sugar is oxidized to a carboxyl group. The oligosaccharide as used in the present invention can be a linear structure or can include branches. The linkage (e.g. glycosidic linkage, galactosidic linkage, glucosidic linkage, etc.) between two sugar units can be expressed, for example, as 1,4, 1->4, or (1-4), used interchangeably herein. For example, the terms "Gal-b1,4-Glc", " β -Gal-(1->4)-Glc", "Galbeta1-4-Glc" and "Gal-b(1-4)-Glc" have the same meaning, i.e. a beta-glycosidic bond links carbon-1 of galactose (Gal) with the carbon-4 of glucose (Glc). Each monosaccharide can be in the cyclic form (e.g. pyranose of furanose form). Linkages between the individual monosaccharide units may include alpha 1->2, alpha 1->3, alpha 1->4, alpha 1->6, alpha 2->1, alpha 2->3, alpha 2->4, alpha 2->6, beta 1->2, beta 1->3, beta 1->4, beta 1->6, beta 2->1, beta 2->3, beta 2->4, and beta 2->6. An oligosaccharide can contain both alpha- and beta-glycosidic bonds or can contain only beta-glycosidic bonds. Preferably, the oligosaccharide as described herein contains monosaccharides selected from the list as used herein below. As used herein, "LNB (lacto-N-biose)-based oligosaccharide" refers to an oligosaccharide as defined herein which contains a LNB at its reducing end. As used herein, "LacNAc (N-acetylglucosamine)-based oligosaccharide" refers to an oligosaccharide as defined herein which contains a LacNAc at its reducing end.

The term "monosaccharide" as used herein refers to a sugar that is not decomposable into simpler sugars by hydrolysis, is classed either an aldose or ketose, and contains one or more hydroxyl groups per molecule. Monosaccharides are saccharides containing only one simple sugar. Examples of monosaccharides comprise Hexose, D-Glucopyranose, D-Galactofuranose, D-Galactopyranose, L-Galactopyranose, D-Mannopyranose, D-Allopyranose, L-Altropyranose, D-Gulopyranose, L-Idopyranose, D-Talopyranose, D-Ribofuranose, D-Ribopyranose, D-Arabinofuranose, D-Arabinopyranose, L-Arabinofuranose, L-Arabinopyranose, D-Xylopyranose, D-Lyxopyranose, D-Erythrofuranose, D-Threofuranose, Heptose, L-glycero-D-manno-Heptopyranose (LDmanHep), D-glycero-D-manno-Heptopyranose (DDmanHep), 6-Deoxy-L-altropyranose, 6-Deoxy-D-gulopyranose, 6-Deoxy-D-

talopyranose, 6-Deoxy-D-galactopyranose, 6-Deoxy-L-galactopyranose, 6-Deoxy-D-mannopyranose, 6-Deoxy-L-mannopyranose, 6-Deoxy-D-glucopyranose, 2-Deoxy-D-arabino-hexose, 2-Deoxy-D-erythro-pentose, 2,6-Dideoxy-D-arabino-hexopyranose, 3,6-Dideoxy-D-arabino-hexopyranose, 3,6-Dideoxy-L-arabino-hexopyranose, 3,6-Dideoxy-D-xylo-hexopyranose, 3,6-Dideoxy-D-ribo-hexopyranose, 2,6-Dideoxy-D-ribo-hexopyranose, 3,6-Dideoxy-L-xylo-hexopyranose, 2-Amino-2-deoxy-D-glucopyranose, 2-Amino-2-deoxy-D-galactopyranose, 2-Amino-2-deoxy-D-mannopyranose, 2-Amino-2-deoxy-D-allopyranose, 2-Amino-2-deoxy-L-altropyranose, 2-Amino-2-deoxy-D-gulopyranose, 2-Amino-2-deoxy-L-idopyranose, 2-Amino-2-deoxy-D-talopyranose, 2-Acetamido-2-deoxy-D-glucopyranose, 2-Acetamido-2-deoxy-D-galactopyranose, 2-Acetamido-2-deoxy-D-mannopyranose, 2-Acetamido-2-deoxy-D-allopyranose, 2-Acetamido-2-deoxy-L-altropyranose, 2-Acetamido-2-deoxy-D-gulopyranose, 2-Acetamido-2-deoxy-L-idopyranose, 2-Acetamido-2-deoxy-D-talopyranose, 2-Acetamido-2,6-dideoxy-D-galactopyranose, 2-Acetamido-2,6-dideoxy-L-galactopyranose, 2-Acetamido-2,6-dideoxy-L-mannopyranose, 2-Acetamido-2,6-dideoxy-D-glucopyranose, 2-Acetamido-2,6-dideoxy-L-altropyranose, 2-Acetamido-2,6-dideoxy-D-talopyranose, D-Glucopyranuronic acid, D-Galactopyranuronic acid, D-Mannopyranuronic acid, D-Allopyranuronic acid, L-Altropyranuronic acid, D-Gulopyranuronic acid, L-Gulopyranuronic acid, L-Idopyranuronic acid, D-Talopyranuronic acid, sialic acid, 5-Amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, 5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, 5-Glycolylamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, Erythritol, Arabinitol, Xylitol, Ribitol, Glucitol, Galactitol, Mannitol, D-ribo-Hex-2-ulopyranose, D-arabino-Hex-2-ulofuranose (D-fructofuranose), D-arabino-Hex-2-ulopyranose, L-xylo-Hex-2-ulopyranose, D-lyxo-Hex-2-ulopyranose, D-threo-Pent-2-ulopyranose, D-altro-Hept-2-ulopyranose, 3-C-(Hydroxymethyl)-D-erythofuranose, 2,4,6-Trideoxy-2,4-diamino-D-glucopyranose, 6-Deoxy-3-O-methyl-D-glucose, 3-O-Methyl-D-rhamnose, 2,6-Dideoxy-3-methyl-D-ribo-hexose, 2-Amino-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 2-Acetamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 2-Glycolylamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 3-Deoxy-D-lyxo-hept-2-ulopyranosaric acid, 3-Deoxy-D-manno-oct-2-ulopyranosonic acid, 3-Deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradeoxy-L-glycero-L-altro-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradeoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradeoxy-D-glycero-D-talo-non-2-ulopyranosonic acid, glucose, galactose, N-acetylglucosamine, glucosamine, mannose, xylose, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid, N-acetylgalactosamine, galactosamine, fucose, rhamnose, glucuronic acid, gluconic acid, fructose and polyols.

With the term polyol is meant an alcohol containing multiple hydroxyl groups. For example, glycerol, sorbitol, or mannitol.

The term "disaccharide" as used herein refers to a saccharide composed of two monosaccharide units. Examples of disaccharides comprise lactose (Gal-b1,4-Glc), lacto-N-biose (Gal-b1,3-GlcNAc), N-

acetyllactosamine (Gal-b1,4-GlcNAc), LacDiNAc (GalNAc-b1,4-GlcNAc), N-acetylgalactosaminylglucose (GalNAc-b1,4-Glc), Neu5Ac-a2,3-Gal, Neu5Ac-a2,6-Gal and fucopyranosyl- (1-4)-N-glycolylneuraminic acid (Fuc-(1-4)-Neu5Gc).

As used herein, "mammalian milk oligosaccharide" (MMO) refers to oligosaccharides such as but not limited to lacto-N-triose II, 3-fucosyllactose, 2'-fucosyllactose, 6-fucosyllactose, 2',3-difucosyllactose, 2',2-difucosyllactose, 3,4-difucosyllactose, 6'-sialyllactose, 3'-sialyllactose, 3,6-disialyllactose, 6,6'-disialyllactose, 8,3-disialyllactose, 3,6-disialyllacto-N-tetraose, lactodifucotetraose, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-fucopentaose II, lacto-N-fucopentaose I, lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-fucopentaose VI, sialyllacto-N-neotetraose d, sialyllacto-N-neotetraose c, sialyllacto-N-tetraose b, sialyllacto-N-tetraose a, lacto-N-difucohexaose I, lacto-N-difucohexaose II, lacto-N-hexaose, lacto-N-neohexaose, para-lacto-N-hexaose, monofucosylmonosialyllacto-N-neotetraose c, monofucosyl para-lacto-N-hexaose, monofucosyllacto-N-hexaose III, isomeric fucosylated lacto-N-hexaose III, isomeric fucosylated lacto-N-hexaose I, sialyllacto-N-hexaose, sialyllacto-N-neohexaose II, difucosyl-para-lacto-N-hexaose, difucosyllacto-N-hexaose, difucosyllacto-N-hexaose a, difucosyllacto-N-hexaose c, galactosylated chitosan, fucosylated oligosaccharides, neutral oligosaccharide and/or sialylated oligosaccharides. Mammalian milk oligosaccharides (MMOs) comprise oligosaccharides present in milk found in any phase during lactation including colostrum milk from humans (i.e. human milk oligosaccharides or HMOs) and mammals including but not limited to cows (*Bos Taurus*), sheep (*Ovis aries*), goats (*Capra aegagrus hircus*), bactrian camels (*Camelus bactrianus*), horses (*Equus ferus caballus*), pigs (*Sus scropha*), dogs (*Canis lupus familiaris*), ezo brown bears (*Ursus arctos yesoensis*), polar bear (*Ursus maritimus*), Japanese black bears (*Ursus thibetanus japonicus*), striped skunks (*Mephitis mephitis*), hooded seals (*Cystophora cristata*), Asian elephants (*Elephas maximus*), African elephant (*Loxodonta africana*), giant anteater (*Myrmecophaga tridactyla*), common bottlenose dolphins (*Tursiops truncatus*), northern minke whales (*Balaenoptera acutorostrata*), tammar wallabies (*Macropus eugenii*), red kangaroos (*Macropus rufus*), common brushtail possum (*Trichosurus Vulpecula*), koalas (*Phascolarctos cinereus*), eastern quolls (*Dasyurus viverrinus*), platypus (*Ornithorhynchus anatinus*). Human milk oligosaccharides (HMOs) are also known as human identical milk oligosaccharides which are chemically identical to the human milk oligosaccharides found in human breast milk but which are biotechnologically-produced (e.g. using cell free systems or cells and organisms comprising a bacterium, a fungus, a yeast, a plant, animal, or protozoan cell, preferably genetically engineered cells and organisms). Human identical milk oligosaccharides are marketed under the name HiMO.

As used herein, "lactose-based mammalian milk oligosaccharide (MMO)" refers to a MMO as defined herein which contains a lactose at its reducing end.

As used herein, a 'sialylated oligosaccharide' is to be understood as a charged sialic acid containing oligosaccharide, i.e. an oligosaccharide having a sialic acid residue. It has an acidic nature. A sialylated oligosaccharide contains at least one sialic acid monosaccharide subunit, like e.g. but not limited to

Neu5Ac, and Neu5Gc. Said sialylated oligosaccharide is a saccharide structure comprising at least three monosaccharide subunits linked to each other via glycosidic bonds, wherein at least one of said monosaccharide subunit is a sialic acid. Said sialylated oligosaccharide can contain more than one sialic acid residue, e.g. two, three or more. Said sialic acid can be linked to other monosaccharide subunits comprising galactose, GlcNAc, sialic acid, via alpha-glycosidic bonds comprising alpha-2,3, alpha-2,6 linkages. Some examples are 3-SL (3'-sialyllactose or 3'-SL or Neu5Ac-a2,3-Gal-b1,4-Glc), 3'-sialyllactosamine, 6-SL (6'-sialyllactose or 6'-SL or Neu5Ac-a2,6-Gal-b1,4-Glc), 6'-sialyllactosamine, oligosaccharides comprising 6'-sialyllactose, 3,6-disialyllactose (Neu5Ac-a2,3-(Neu5Ac-a2,6)-Gal-b1,4-Glc), 6,6'-disialyllactose (Neu5Ac-a2,6-Gal-b1,4-(Neu5Ac-a2,6)-Glc), 8,3-disialyllactose (Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc), SGG hexasaccharide (Neu5Ac-a2,3Gal β -1,3GalNAc β -1,3Gal α -1,4Gal β -1,4Gal), sialylated tetrasaccharide (Neu5Ac-a2,3Gal β -1,4GlcNAc β -1,4GlcNAc), pentasaccharide LSTD (Neu5Ac-a2,3Gal β -1,4GlcNAc β -1,3Gal β -1,4Glc), sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialyllacto-N-neotetraose, monosialyllacto-N-hexaose, disialyllacto-N-hexaose I, monosialyllacto-N-neohexaose I, monosialyllacto-N-neohexaose II, disialyllacto-N-neohexaose, disialyllacto-N-tetraose, disialyllacto-N-hexaose II, sialyllacto-N-tetraose a, disialyllacto-N-hexaose I, sialyllacto-N-tetraose b, sialyllacto-N-neotetraose c, sialyllacto-N-neotetraose d, 3'-sialyl-3-fucosyllactose, disialomonofucosyllacto-N-neohexaose, monofucosylmonosialyllacto-N-octaose (sialyl Lea), sialyllacto-N-fucohexaose II, disialyllacto-N-fucopentaose II, monofucosyldisialyllacto-N-tetraose and oligosaccharides bearing one or several sialic acid residu(s), including but not limited to: oligosaccharide moieties of the gangliosides selected from GM3 (3'-sialyllactose, Neu5Ac-a2,3Gal β -4Glc) and oligosaccharides comprising the GM3 motif, GD3 Neu5Ac-a2,8Neu5Ac-a2,3Gal β -1,4Glc GT3 (Neu5Ac-a2,8Neu5Ac-a2,8Neu5Ac-a2,3Gal β -1,4Glc); GM2 GalNAc β -1,4(Neu5Ac-a2,3)Gal β -1,4Glc, GM1 Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,3)Gal β -1,4Glc, GD1a Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,3)Gal β -1,4Glc, GT1a Neu5Ac-a2,8Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,3)Gal β -1,4Glc, GD2 GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GT2 GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GD1b, Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GT1b Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GQ1b Neu5Ac-a2,8Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GT1c Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GQ1c Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GP1c Neu5Ac-a2,8Neu5Ac-a2,3Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,8Neu5Ac-a2,8Neu5Ac-a2,3)Gal β -1,4Glc, GD1a Neu5Ac-a2,3Gal β -1,3(Neu5Ac-a2,6)GalNAc β -1,4Gal β -1,4Glc, Fucosyl-GM1 Fuca-1,2Gal β -1,3GalNAc β -1,4(Neu5Ac-a2,3)Gal β -1,4Glc; all of which may be extended to the production of the corresponding gangliosides by reacting the above oligosaccharide moieties with ceramide or synthesizing the above oligosaccharides on a ceramide.

The terms "alpha-2,3-sialyltransferase", "alpha 2,3 sialyltransferase", "3-sialyltransferase", "α-2,3-sialyltransferase", "α 2,3 sialyltransferase", "3 sialyltransferase", "3-ST" or "3ST" as used in the present

invention, are used interchangeably and refer to a glycosyltransferase that catalyzes the transfer of sialic acid from the donor CMP-Neu5Ac, to the acceptor molecule in an alpha-2,3-linkage. The terms "3' sialyllactose", "3'-sialyllactose", "alpha-2,3-sialyllactose", "alpha 2,3 sialyllactose", "α-2,3-sialyllactose", "α 2,3 sialyllactose", "3SL" or "3'SL" as used in the present invention, are used interchangeably and refer to the product obtained by the catalysis of the alpha-2,3-fucosyltransferase transferring the sialic acid group from CMP-Neu5Ac to lactose in an alpha-2,3-linkage. The terms "alpha-2,6-sialyltransferase", "alpha 2,6 sialyltransferase", "6-sialyltransferase", "α-2,6-sialyltransferase", "α 2,6 sialyltransferase", "6 sialyltransferase", "6-ST" or "6ST" as used in the present invention, are used interchangeably and refer to a glycosyltransferase that catalyzes the transfer of sialic acid from the donor CMP-Neu5Ac, to the acceptor molecule in an alpha-2,6-linkage. The terms "6' sialyllactose", "6'-sialyllactose", "alpha-2,6-sialyllactose", "alpha 2,6 sialyllactose", "α-2,6-sialyllactose", "α 2,6 sialyllactose", "6SL" or "6'SL" as used in the present invention, are used interchangeably and refer to the product obtained by the catalysis of the alpha-2,6-fucosyltransferase transferring the sialic acid group from CMP-Neu5Ac to lactose in an alpha-2,6-linkage. The terms "alpha-2,8-sialyltransferase", "alpha 2,8 sialyltransferase", "8-sialyltransferase", "α-2,8-sialyltransferase", "α 2,8 sialyltransferase", "8 sialyltransferase", "8-ST" or "8ST" as used in the present invention, are used interchangeably and refer to a glycosyltransferase that catalyzes the transfer of sialic acid from the donor CMP-Neu5Ac, to the acceptor in an alpha-2,8-linkage.

A 'fucosylated oligosaccharide' as used herein and as generally understood in the state of the art is an oligosaccharide that is carrying a fucose-residue. Such fucosylated oligosaccharide is a saccharide structure comprising at least three monosaccharide subunits linked to each other via glycosidic bonds, wherein at least one of said monosaccharide subunit is a fucose. A fucosylated oligosaccharide can contain more than one fucose residue, e.g. two, three or more. A fucosylated oligosaccharide can be a neutral oligosaccharide or a charged oligosaccharide e.g. also comprising sialic acid structures. Fucose can be linked to other monosaccharide subunits comprising glucose, galactose, GlcNAc via alpha-glycosidic bonds comprising alpha-1,2 alpha-1,3, alpha-1,4, alpha-1,6 linkages. Examples comprise 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 4-fucosyllactose (4FL), 6-fucosyllactose (6FL), difucosyllactose (diFL), lactodifucotetraose (LDFT), Lacto-N-fucopentaose I (LNFP I), Lacto-N-fucopentaose II (LNFP II), Lacto-N-fucopentaose III (LNFP III), lacto-N-fucopentaose V (LNFP V), lacto-N-fucopentaose VI (LNFP VI), lacto-N-neofucopentaose I, lacto-N-difucohexaose I (LDFH I), lacto-N-difucohexaose II (LDFH II), Monofucosyllacto-N-hexaose III (MFLNH III), Difucosyllacto-N-hexaose (DFLNHa), difucosyl-lacto-N-neohexaose, 3'-sialyl-3-fucosyllactose, disialomonofucosyllacto-N-neohexaose, monofucosylmonosialyllacto-N-octaose (sialyl Lea), sialyllacto-N-fucohexaose II, disialyllacto-N-fucopentaose II, monofucosyldisialyllacto-N-tetraose.

The terms "alpha-1,2-fucosyltransferase", "alpha 1,2 fucosyltransferase", "2-fucosyltransferase", "α-1,2-fucosyltransferase", "α 1,2 fucosyltransferase", "2 fucosyltransferase", "2-FT" or "2FT" as used in the present invention, are used interchangeably and refer to a glycosyltransferase that catalyzes the transfer

of fucose from the donor GDP-L-fucose, to the acceptor molecule in an alpha-1,2-linkage. The terms "2'-fucosyllactose", "2'-fucosyllactose", "alpha-1,2-fucosyllactose", "alpha 1,2 fucosyllactose", "α-1,2-fucosyllactose", "α 1,2 fucosyllactose", "Galβ-4(Fucα1-2)Glc", 2FL" or "2'FL" as used in the present invention, are used interchangeably and refer to the product obtained by the catalysis of the alpha-1,2-fucosyltransferase transferring the fucose residue from GDP-L-fucose to lactose in an alpha-1,2-linkage.

The terms "difucosyllactose", "di-fucosyllactose", "lactodifucotetraose", "2',3-difucosyllactose", "2',3-difucosyllactose", "α-2',3-fucosyllactose", "α 2',3 fucosyllactose", "Fucα1-2Galβ 1-4(Fucα1-3)Glc", "DFLac", 2',3 diFL", "DFL", "DiFL" or "diFL" as used in the present invention, are used interchangeably.

The terms "alpha-1,3-fucosyltransferase", "alpha 1,3 fucosyltransferase", "3-fucosyltransferase", "α-1,3-fucosyltransferase", "α 1,3 fucosyltransferase", "3 fucosyltransferase", "3-FT" or "3FT" as used in the present invention, are used interchangeably and refer to a glycosyltransferase that catalyzes the transfer of fucose from the donor GDP-L-fucose, to the acceptor molecule in an alpha-1,3-linkage. The terms "3-fucosyllactose", "alpha-1,3-fucosyllactose", "alpha 1,3 fucosyllactose", "α-1,3-fucosyllactose", "α 1,3 fucosyllactose", "Galβ-4(Fucα1-3)Glc", 3FL" or "3-FL" as used in the present invention, are used interchangeably and refer to the product obtained by the catalysis of the alpha-1,3-fucosyltransferase transferring the fucose residue from GDP-L-fucose to lactose in an alpha-1,3-linkage.

A 'neutral oligosaccharide' as used herein and as generally understood in the state of the art is an oligosaccharide that has no negative charge originating from a carboxylic acid group. Examples of such neutral oligosaccharide are 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 2', 3-difucosyllactose (diFL), lacto-N-triose II, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-fucopentaose I, lacto-N-neofucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-fucopentaose VI, lacto-N-neofucopentaose V, lacto-N-difucohexaose I, lacto-N-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-N-hexaose, lacto-N-neohexaose, para-lacto-N-hexaose, para-lacto-N-neohexaose, difucosyl-lacto-N-hexaose and difucosyl-lacto-N-neohexaose.

The terms "LNB" and "Lacto-N-biose" are used interchangeably and refer to the disaccharide Gal-b1,3-GlcNAc.

The terms "LacNAc" and "N-acetyllactosamine" are used interchangeably and refer to the disaccharide Gal-b1,4-GlcNAc.

The terms "LNT II", "LNT-II", "LN3", "lacto-N-triose II", "lacto-N-triose II", "lacto-N-triose", "lacto-N-triose" or "GlcNAcβ1-3Galβ1-4Glc" as used in the present invention, are used interchangeably.

The terms "LNT", "lacto-N-tetraose", "lacto-N-tetraose" or "Galβ1-3GlcNAcβ1-3Galβ1-4Glc" as used in the present invention, are used interchangeably.

The terms "LNnT", "lacto-N-neotetraose", "lacto-N-neotetraose", "neo-LNT" or "Galβ1-4GlcNAcβ1-3Galβ1-4Glc" as used in the present invention, are used interchangeably.

The terms "LSTa", "LS-Tetrasaccharide a", "Sialyl-lacto-N-tetraose a", "sialyllacto-N-tetraose a" or "Neu5Ac-a2,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc" as used in the present invention, are used

interchangeably.

The terms "LSTb", "LS-Tetrasaccharide b", "Sialyl-lacto-N-tetraose b", "sialyllacto-N-tetraose b" or "Gal-b1,3-(Neu5Ac-a2,6)-GlcNAc-b1,3-Gal-b1,4-Glc" as used in the present invention, are used interchangeably.

- 5 The terms "LSTc", "LS-Tetrasaccharide c", "Sialyl-lacto-N-tetraose c", "sialyllacto-N-tetraose c", "sialyllacto-N-neotetraose c" or "Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc" as used in the present invention, are used interchangeably.

- The terms "LSTd", "LS-Tetrasaccharide d", "Sialyl-lacto-N-tetraose d", "sialyllacto-N-tetraose d", "sialyllacto-N-neotetraose d" or "Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc" as used in the present
10 invention, are used interchangeably.

The terms "DSLNNt" and "Disialyllacto-N-neotetraose" are used interchangeably and refer to Neu5Ac-a2,6-[Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,3]-Gal-b1,4-Glc.

- The terms "DSLNT" and "Disialyllacto-N-tetraose" are used interchangeably and refer to Neu5Ac-a2,6-[Neu5Ac-a2,3-Gal-b1,3-GlcNAc-b1,3]-Gal-b1,4-Glc. The terms "LNFP-I", "lacto-N-fucopentaose I", "LNFP
15 I", "LNF I OH type I determinant", "LNF I", "LNF1", "LNF 1" and "Blood group H antigen pentose type 1" are used interchangeably and refer to Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc.

The terms "GalNAc-LNFP-I" and "blood group A antigen hexose type I" are used interchangeably and refer to GalNAc-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc.

- The terms "LNFP-II" and "lacto-N-fucopentaose II" are used interchangeably and refer to Gal-b1,3-(Fuc-a1,4)-GlcNAc-b1,3-Gal-b1,4-Glc.
20

The terms "LNFP-III" and "lacto-N-fucopentaose III" are used interchangeably and refer to Gal-b1,4-(Fuc-a1,3)-GlcNAc-b1,3-Gal-b1,4-Glc.

The terms "LNFP-V" and "lacto-N-fucopentaose V" are used interchangeably and refer to Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-(Fuc-a1,3)-Glc.

- 25 The terms "LNFP-VI", "LNnFP V" and "lacto-N-neofucopentaose V" are used interchangeably and refer to Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-(Fuc-a1,3)-Glc.

The terms "LNnFP I" and "Lacto-N-neofucopentaose I" are used interchangeably and refer to Fuc-a1,2-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc.

- The terms "LNDFH I", "Lacto-N-difucohexaose I", "LNDFH-I", "LDFH I", "Le^b-lactose" and "Lewis-b
30 hexasaccharide" are used interchangeably and refer to Fuc-a1,2-Gal-b1,3-[Fuc-a1,4]-GlcNAc-b1,3-Gal-b1,4-Glc.

The terms "LNDFH II", "Lacto-N-difucohexaose II", "Lewis a-Lewis x" and "LDFH II" are used interchangeably and refer to Fuc-a1,4-(Gal-b1,3)-GlcNAc-b1,3-Gal-b1,4-(Fuc-a1,3)-Glc.

- The terms "LNnDFH", "Lacto-N-neoDiFucohexaose" and "Lewis x hexaose" are used interchangeably and
35 refer to Gal-b1,4-(Fuc-a1,3)-GlcNAc-b1,3-Gal-b1,4-(Fuc-a1,3)-Glc.

The terms "alpha-tetrasaccharide" and "A-tetrasaccharide" are used interchangeably and refer to GalNAc-

a1,3-(Fuc-a1,2)-Gal-b1,4-Glc.

A 'fucosylation pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase and/or the salvage pathway L-fucokinase/GDP-fucose pyrophosphorylase, combined with a fucosyltransferase leading to α 1,2; α 1,3 α 1,4 and/or α 1,6 fucosylated oligosaccharides.

A 'sialylation pathway' is a biochemical pathway consisting of the enzymes and their respective genes, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acetylglucosamine epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylglucosamine-6P 2-epimerase, Glucosamine 6-phosphate N-acetyltransferase, N-AcetylGlucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridyltransferase, glucosamine-1-phosphate acetyltransferase, sialic acid synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, and/or CMP-sialic acid synthase, combined with a sialyltransferase leading to α 2,3; α 2,6 and/or α 2,8 sialylated oligosaccharides.

A 'galactosylation pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, and/or glucophosphomutase, combined with a galactosyltransferase leading to an alpha or beta bound galactose on the 2, 3, 4, and/or 6 hydroxyl group of an oligosaccharide.

An 'N-acetylglucosamine carbohydrate pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, and/or glucosamine-1-phosphate acetyltransferase, combined with a glycosyltransferase leading to an alpha or beta bound N-acetylglucosamine on the 3, 4, and/or 6 hydroxyl group of an oligosaccharide.

An 'N-acetylgalactosaminylation pathway' as used herein is a biochemical pathway comprising at least one of the enzymes and their respective genes chosen from the list comprising L-glutamine—D-fructose-6-phosphate aminotransferase, phosphoglucosamine mutase, N-acetylglucosamine 1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, UDP-N-acetylglucosamine 4-epimerase, UDP-glucose 4-epimerase, N-acetylgalactosamine kinase and/or UDP-N-acetylgalactosamine pyrophosphorylase combined with a glycosyltransferase leading to a GalNAc-modified compound comprising a mono-, di- or oligosaccharide having an alpha or beta bound N-acetylgalactosamine on said mono-, di- or oligosaccharide.

A 'mannosylation pathway' as used herein is a biochemical pathway comprising at least one of the enzymes and their respective genes chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase and/or mannose-1-phosphate guanylyltransferase combined with a glycosyltransferase leading to a mannosylated compound comprising a mono-, di- or oligosaccharide having an alpha or beta bound mannose on said mono-, di- or oligosaccharide.

An 'N-acetylmannosaminylation pathway' as used herein is a biochemical pathway comprising at least one of the enzymes and their respective genes chosen from the list comprising L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-1-phosphate uridyltransferase, glucosamine-1-phosphate acetyltransferase, glucosamine-1-phosphate acetyltransferase, UDP-GlcNAc 2-epimerase and/or ManNAc kinase combined with a glycosyltransferase leading to a ManNAc-modified compound comprising a mono-, di- or oligosaccharide having an alpha or beta bound N-acetylmannosamine on said mono-, di- or oligosaccharide.

The terms "mannose-6-phosphate isomerase", "phosphomannose isomerase", "mannose phosphate isomerase", "phosphohexoisomerase", "phosphomannoisomerase", "phosphomannose-isomerase", "phosphohexomutase", "D-mannose-6-phosphate ketol-isomerase" and "manA" are used interchangeably and refer to an enzyme that catalyses the reversible conversion of D-fructose 6-phosphate to D-mannose 6-phosphate.

The terms "phosphomannomutase", "mannose phosphomutase", "phosphomannose mutase", "D-mannose 1,6-phosphomutase" and "manB" are used interchangeably and refer to an enzyme that catalyses the reversible conversion of D-mannose 6-phosphate to D-mannose 1-phosphate.

The terms "mannose-1-phosphate guanylyltransferase", "GTP-mannose-1-phosphate guanylyltransferase", "PIM-GMP (phosphomannose isomerase-guanosine 5'-diphospho-D-mannose pyrophosphorylase)", "GDP-mannose pyrophosphorylase", "guanosine 5'-diphospho-D-mannose pyrophosphorylase", "guanosine diphosphomannose pyrophosphorylase", "guanosine triphosphate-mannose 1-phosphate guanylyltransferase", "mannose 1-phosphate guanylyltransferase (guanosine triphosphate)" and "manC" are used interchangeably and refer to an enzyme that converts D-mannose-1-phosphate using GTP into GDP-mannose and diphosphate.

The terms "GDP-mannose 4,6-dehydratase", "guanosine 5'-diphosphate-D-mannose oxidoreductase", "guanosine diphosphomannose oxidoreductase", "guanosine diphosphomannose 4,6-dehydratase", "GDP-D-mannose dehydratase", "GDP-D-mannose 4,6-dehydratase", "GDP-mannose 4,6-hydro-lyase", "GDP-mannose 4,6-hydro-lyase (GDP-4-dehydro-6-deoxy-D-mannose-forming)" and "gmd" are used interchangeably and refer to an enzyme that forms the first step in the biosynthesis of GDP-rhamnose and GDP-fucose.

The terms "GDP-L-fucose synthase", "GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase",

"GDP-L-fucose:NADP+ 4-oxidoreductase (3,5-epimerizing)" and "fcl" are used interchangeably and refer to an enzyme that forms the second step in the biosynthesis of GDP-fucose.

The terms "L-fucokinase/GDP-fucose pyrophosphorylase", "L-fucokinase/L-fucose-1-P guanylyltransferase", "GDP-fucose pyrophosphorylase", "GDP-L-fucose pyrophosphorylase", and "fcp" are used interchangeably and refer to an enzyme that catalyses the conversion of L-fucose-1-phosphate into GDP-fucose using GTP.

The terms "L-glutamine—D-fructose-6-phosphate aminotransferase", "glutamine---fructose-6-phosphate transaminase (isomerizing)", "hexosephosphate aminotransferase", "glucosamine-6-phosphate isomerase (glutamine-forming)", "glutamine-fructose-6-phosphate transaminase (isomerizing)", "D-fructose-6-phosphate amidotransferase", "fructose-6-phosphate aminotransferase", "glucosaminephosphate isomerase", "glucosamine 6-phosphate synthase", "GlcN6P synthase", "GFA", "glms", "glmS" and "glmS*54" are used interchangeably and refer to an enzyme that catalyses the conversion of D-fructose-6-phosphate into D-glucosamine-6-phosphate using L-glutamine.

The terms "glucosamine-6-P deaminase", "glucosamine-6-phosphate deaminase", "GlcN6P deaminase", "glucosamine-6-phosphate isomerase", "glmD" and "nagB" are used interchangeably and refer to an enzyme that catalyses the reversible isomerization-deamination of glucosamine-6-phosphate (GlcN6P) to form fructose-6-phosphate and an ammonium ion.

The terms "phosphoglucosamine mutase" and "glmM" are used interchangeably and refer to an enzyme that catalyses the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate. Phosphoglucosamine mutase can also catalyse the formation of glucose-6-P from glucose-1-P, although at a 1400-fold lower rate.

The terms "N-acetylglucosamine-6-P deacetylase", "N-acetylglucosamine-6-phosphate deacetylase" and "nagA" are used interchangeably and refer to an enzyme that catalyses the hydrolysis of the N-acetyl group of N-acetylglucosamine-6-phosphate (GlcNAc-6-P) to yield glucosamine-6-phosphate (GlcN6P) and acetate.

An N-acylglucosamine 2-epimerase is an enzyme that catalyses the reaction N-acyl-D-glucosamine = N-acyl-D-mannosamine. Alternative names for this enzyme comprise N-acetylglucosamine 2-epimerase, N-acetyl-D-glucosamine 2-epimerase, GlcNAc 2-epimerase, N-acyl-D-glucosamine 2-epimerase and N-acetylglucosamine epimerase.

An UDP-N-acetylglucosamine 2-epimerase is an enzyme that catalyses the reaction N-acetyl-D-glucosamine = N-acetylmannosamine. Alternative names for this enzyme comprise UDP-N-acylglucosamine 2-epimerase, UDP-GlcNAc-2-epimerase and UDP-N-acetyl-D-glucosamine 2-epimerase.

An N-acetylmannosamine-6-phosphate 2-epimerase is an enzyme that catalyses the reaction N-acetyl-D-glucosamine 6-phosphate = N-acetyl-D-mannosamine 6-phosphate.

A bifunctional UDP-GlcNAc 2-epimerase/kinase is a bifunctional enzyme that catalyses the reaction UDP-N-acetyl-D-glucosamine = N-acetyl-D-mannosamine and the reaction N-acetyl-D-mannosamine + ATP =

ADP + N-acetyl-D-mannosamine 6-phosphate.

A glucosamine 6-phosphate N-acetyltransferase is an enzyme that catalyses the transfer of an acetyl group from acetyl-CoA to D-glucosamine-6-phosphate thereby generating a free CoA and N-acetyl-D-glucosamine 6-phosphate. Alternative names comprise aminodeoxyglucosephosphate acetyltransferase, D-glucosamine-6-P N-acetyltransferase, glucosamine 6-phosphate acetylase, glucosamine 6-phosphate N-acetyltransferase, glucosamine-phosphate N-acetyltransferase, glucosamine-6-phosphate acetylase, N-acetylglucosamine-6-phosphate synthase, phosphoglucosamine acetylase, phosphoglucosamine N-acetylase phosphoglucosamine N-acetylase, phosphoglucosamine transacetylase, GNA and GNA1.

The term "N-acetylglucosamine-6-phosphate phosphatase" refers to an enzyme that dephosphorylates N-acetylglucosamine-6-phosphate (GlcNAc-6-P) hereby synthesizing N-acetylglucosamine (GlcNAc).

The term "N-acetylmannosamine-6-phosphate phosphatase" refers to an enzyme that dephosphorylates N-acetylmannosamine-6-phosphate (ManNAc-6P) to N-acetylmannosamine (ManNAc).

The terms "N-acetylmannosamine-6-phosphate 2-epimerase", "ManNAc-6-P isomerase", "ManNAc-6-P 2-epimerase", N-acetylglucosamine-6P 2-epimerase and "nanE" are used interchangeably and refer to an enzyme that converts ManNAc-6-P to N-acetylglucosamine-6-phosphate (GlcNAc-6-P).

The terms "phosphoacetylglucosamine mutase", "acetylglucosamine phosphomutase", "acetylaminodeoxyglucose phosphomutase", "phospho-N-acetylglucosamine mutase" and "N-acetyl-D-glucosamine 1,6-phosphomutase" are used interchangeably and refer to an enzyme that catalyses the conversion of N-acetyl-glucosamine 1-phosphate into N-acetylglucosamine 6-phosphate.

The terms "N-acetylglucosamine 1-phosphate uridylyltransferase", "N-acetylglucosamine-1-phosphate uridylyltransferase", "UDP-N-acetylglucosamine diphosphorylase", "UDP-N-acetylglucosamine pyrophosphorylase", "uridine diphosphoacetylglucosamine pyrophosphorylase", "UTP:2-acetamido-2-deoxy-alpha-D-glucose-1-phosphate uridylyltransferase", "UDP-GlcNAc pyrophosphorylase", "GlmU uridylyltransferase", "Acetylglucosamine 1-phosphate uridylyltransferase", "UDP-acetylglucosamine pyrophosphorylase", "uridine diphosphate-N-acetylglucosamine pyrophosphorylase", "uridine diphosphoacetylglucosamine phosphorylase", and "acetylglucosamine 1-phosphate uridylyltransferase" are used interchangeably and refer to an enzyme that catalyses the conversion of N-acetylglucosamine 1-phosphate (GlcNAc-1-P) into UDP-N-acetylglucosamine (UDP-GlcNAc) by the transfer of uridine 5-monophosphate (from uridine 5-triphosphate (UTP)).

The term glucosamine-1-phosphate acetyltransferase refers to an enzyme that catalyses the transfer of the acetyl group from acetyl coenzyme A to glucosamine-1-phosphate (GlcN-1-P) to produce N-acetylglucosamine-1-phosphate (GlcNAc-1-P).

The term "glmU" refers to a bifunctional enzyme that has both N-acetylglucosamine-1-phosphate uridylyltransferase and glucosamine-1-phosphate acetyltransferase activity and that catalyses two sequential reactions in the de novo biosynthetic pathway for UDP-GlcNAc. The C-terminal domain catalyses the transfer of acetyl group from acetyl coenzyme A to GlcN-1-P to produce GlcNAc-1-P, which

is converted into UDP-GlcNAc by the transfer of uridine 5-monophosphate, a reaction catalysed by the N-terminal domain.

The terms "NeunAc synthase", "N-acetylneuraminic acid synthase", "N-acetylneuraminate synthase", "sialic acid synthase", "NeuAc synthase", "NeuB", "NeuB1", "NeuNAc synthase", "NANA condensing enzyme", "N-acetylneuraminate lyase synthase", "N-acetylneuraminic acid condensing enzyme" as used
5 herein are used interchangeably and refer to an enzyme capable to synthesize sialic acid from N-acetylmannosamine (ManNAc) in a reaction using phosphoenolpyruvate (PEP).

The terms "N-acetylneuraminate lyase", "Neu5Ac lyase", "N-acetylneuraminate pyruvate-lyase", "N-acetylneuraminic acid aldolase", "NALase", "sialate lyase", "sialic acid aldolase", "sialic acid lyase" and
10 "nanA" are used interchangeably and refer to an enzyme that degrades N-acetylneuraminate into N-acetylmannosamine (ManNAc) and pyruvate.

The terms "N-acylneuraminate-9-phosphate synthase", "N-acylneuraminate-9-phosphate synthetase", "NANA synthase", "NANAS", "NANS", "NmeNANAS", "N-acetylneuraminate pyruvate-lyase (pyruvate-phosphorylating)" as used herein are used interchangeably and refer to an enzyme capable to synthesize
15 N-acylneuraminate-9-phosphate from N-acetylmannosamine-6-phosphate (ManNAc-6-phosphate) in a reaction using phosphoenolpyruvate (PEP).

The term "N-acylneuraminate-9-phosphatase" refers to an enzyme capable to dephosphorylate N-acylneuraminate-9-phosphate to synthesise N-acylneuraminate.

The terms "CMP-sialic acid synthase", "N-acylneuraminate cytidyltransferase", "CMP-sialate synthase",
20 "CMP-NeuAc synthase", "NeuA" and "CMP-N-acetylneuraminic acid synthase" as used herein are used interchangeably and refer to an enzyme capable to synthesize CMP-N-acetylneuraminate from N-acetylneuraminate using CTP in the reaction.

The terms "galactose-1-epimerase", "aldose 1-epimerase", "mutarotase", "aldose mutarotase", "galactose mutarotase", "galactose 1-epimerase" and "D-galactose 1-epimerase" are used
25 interchangeably and refer to an enzyme that catalyses the conversion of beta-D-galactose into alpha-D-galactose.

The terms "galactokinase", "galactokinase (phosphorylating)" and "ATP:D-galactose-1-phosphotransferase" are used interchangeably and refer to an enzyme that catalyses the conversion of
alpha-D-galactose into alpha-D-galactose 1-phosphate using ATP.

The terms glucokinase, and "glucokinase (phosphorylating)" are used interchangeably and refer to an
30 enzyme that catalyses the conversion of D-glucose into D-glucose 6-phosphate using ATP.

The terms "galactose-1-phosphate uridylyltransferase", "Gal-1-P uridylyltransferase", "UDP-glucose---hexose-1-phosphate uridylyltransferase", "uridyl transferase", "hexose-1-phosphate uridylyltransferase", "uridylyltransferase"; "hexose 1-phosphate uridylyltransferase", "UDP-glucose:alpha-D-galactose-1-
35 phosphate uridylyltransferase", "galB" and "galT" are used interchangeably and refer to an enzyme that catalyses the reaction $D\text{-galactose 1-phosphate} + \text{UDP-D-glucose} = D\text{-glucose 1-phosphate} + \text{UDP-D-}$

galactose.

The terms "UDP-glucose 4-epimerase", "UDP-galactose 4-epimerase", "uridine diphosphoglucose epimerase", "galactowaldenase", "UDPG-4-epimerase", "uridine diphosphate galactose 4-epimerase", "uridine diphospho-galactose-4-epimerase", "UDP-glucose epimerase", "4-epimerase", "uridine diphosphoglucose 4-epimerase", "uridine diphosphate glucose 4-epimerase" and "UDP-D-galactose 4-epimerase" are used interchangeably and refer to an enzyme that catalyses the conversion of UDP-D-glucose into UDP-galactose.

The terms "glucose-1-phosphate uridylyltransferase", "UTP---glucose-1-phosphate uridylyltransferase", "UDP glucose pyrophosphorylase", "UDPG phosphorylase", "UDPG pyrophosphorylase", "uridine 5'-diphosphoglucose pyrophosphorylase", "uridine diphosphoglucose pyrophosphorylase", "uridine diphosphate-D-glucose pyrophosphorylase", "uridine-diphosphate glucose pyrophosphorylase" and "galU" are used interchangeably and refer to an enzyme that catalyses the conversion of D-glucose-1-phosphate into UDP-glucose using UTP.

The terms "phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)", "glucose phosphomutase (ambiguous)" and "phosphoglucose mutase (ambiguous)" are used interchangeably and refer to an enzyme that catalyses the conversion of D-glucose 1-phosphate into D-glucose 6-phosphate.

The terms "UDP-N-acetylglucosamine 4-epimerase", "UDP acetylglucosamine epimerase", "uridine diphosphoacetylglucosamine epimerase", "uridine diphosphate N-acetylglucosamine-4-epimerase", "uridine 5'-diphospho-N-acetylglucosamine-4-epimerase" and "UDP-N-acetyl-D-glucosamine 4-epimerase" are used interchangeably and refer to an enzyme that catalyses the epimerization of UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-N-acetylgalactosamine (UDP-GalNAc).

The terms "N-acetylgalactosamine kinase", "GALK2", "GK2", "GalNAc kinase", "N-acetylgalactosamine (GalNAc)-1-phosphate kinase" and "ATP:N-acetyl-D-galactosamine 1-phosphotransferase" are used interchangeably and refer to an enzyme that catalyses the synthesis of N-acetylgalactosamine 1-phosphate (GalNAc-1-P) from N-acetylgalactosamine (GalNAc) using ATP.

The terms "UDP-N-acetylgalactosamine pyrophosphorylase" and "UDP-GalNAc pyrophosphorylase" are used interchangeably and refer to an enzyme that catalyses the conversion of N-acetylgalactosamine 1-phosphate (GalNAc-1-P) into UDP-N-acetylgalactosamine (UDP-GalNAc) using UTP.

The terms "N-acetylneuraminate kinase", "ManNAc kinase", "N-acetyl-D-mannosamine kinase" and "nanK" are used interchangeably and refer to an enzyme that phosphorylates ManNAc to synthesize N-acetylmannosamine-phosphate (ManNAc-6-P).

The terms "acetyl-coenzyme A synthetase", "acs", "acetyl-CoA synthetase", "AcCoA synthetase", "acetate--CoA ligase", "acyl-activating enzyme" and "yfaC" are used interchangeably and refer to an enzyme that catalyses the conversion of acetate into acetyl-coenzyme A (AcCoA) in an ATP-dependent reaction.

The terms "pyruvate dehydrogenase", "pyruvate oxidase", "POX", "poxB" and "pyruvate:ubiquinone-8

oxidoreductase" are used interchangeably and refer to an enzyme that catalyses the oxidative decarboxylation of pyruvate to produce acetate and CO₂.

The terms "lactate dehydrogenase", "D-lactate dehydrogenase", "ldhA", "hslI", "htpH", "D-LDH", "fermentative lactate dehydrogenase" and "D-specific 2-hydroxyacid dehydrogenase" are used interchangeably and refer to an enzyme that catalyses the conversion of lactate into pyruvate hereby generating NADH.

As used herein, the term "cell productivity index (CPI)" refers to the mass of the product produced by the cells divided by the mass of the cells produced in the culture.

The term "purified" refers to material that is substantially or essentially free from components which interfere with the activity of the biological molecule. For cells, saccharides, nucleic acids, and polypeptides, the term "purified" refers to material that is substantially or essentially free from components which normally accompany the material as found in its native state. Typically, purified saccharides, oligosaccharides, proteins or nucleic acids of the invention are at least about 50 %, 55 %, 60 %, 65 %, 70 %, 75 %, 80 % or 85 % pure, usually at least about 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, or 99 % pure as measured by band intensity on a silver-stained gel or other method for determining purity. Purity or homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein or nucleic acid sample, followed by visualization upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized. For oligosaccharides, purity can be determined using methods such as but not limited to thin layer chromatography, gas chromatography, NMR, HPLC, capillary electrophoresis or mass spectroscopy.

The terms "identical" or "percent identity" or "% identity" in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using sequence comparison algorithms or by visual inspection. For sequence comparison, one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Percent identity may be calculated globally over the full-length sequence of the reference sequence, resulting in a global percent identity score. Alternatively, percent identity may be calculated over a partial sequence of the reference sequence, resulting in a local percent identity score. Using the full-length of the reference sequence in a local sequence alignment results in a global percent identity score between the test and the reference sequence. Percent identity can be determined using different algorithms like for example BLAST and PSI-BLAST (Altschul *et al.*, 1990, J Mol

Biol 215:3, 403- 410; Altschul *et al.*, 1997, Nucleic Acids Res 25: 17, 3389-402), the Clustal Omega method (Sievers *et al.*, 2011, Mol. Syst. Biol. 7:539), the MatGAT method (Campanella *et al.*, 2003, BMC Bioinformatics, 4:29) or EMBOSS Needle.

The BLAST (Basic Local Alignment Search Tool) method of alignment is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare sequences using default parameters. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool) derives a position-specific scoring matrix (PSSM) or profile from the multiple sequence alignment of sequences detected above a given score threshold using protein-protein BLAST (BLASTp). The BLAST method can be used for pairwise or multiple sequence alignments. Pairwise Sequence Alignment is used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between two biological sequences (protein or nucleic acid). The web interface for BLAST is available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Clustal Omega (Clustal W) is a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. It produces biologically meaningful multiple sequence alignments of divergent sequences. The web interface for Clustal W is available at <https://www.ebi.ac.uk/Tools/msa/clustalo/>. Default parameters for multiple sequence alignments and calculation of percent identity of protein sequences using the Clustal W method are: enabling de-alignment of input sequences: FALSE; enabling mbed-like clustering guide-tree: TRUE; enabling mbed-like clustering iteration: TRUE; Number of (combined guide-tree/HMM) iterations: default(0); Max Guide Tree Iterations: default [-1]; Max HMM Iterations: default [-1]; order: aligned.

MatGAT (Matrix Global Alignment Tool) is a computer application that generates similarity/identity matrices for DNA or protein sequences without needing pre-alignment of the data. The program performs a series of pairwise alignments using the Myers and Miller global alignment algorithm, calculates similarity and identity, and then places the results in a distance matrix. The user may specify which type of alignment matrix (e.g. BLOSUM50, BLOSUM62, and PAM250) to employ with their protein sequence examination.

EMBOSS Needle (<https://galaxy-iuc.github.io/emboss-5.0-docs/needle.html>) uses the Needleman-Wunsch global alignment algorithm to find the optimal alignment (including gaps) of two sequences when considering their entire length. The optimal alignment is ensured by dynamic programming methods by exploring all possible alignments and choosing the best. The Needleman-Wunsch algorithm is a member of the class of algorithms that can calculate the best score and alignment in the order of mn steps, (where 'n' and 'm' are the lengths of the two sequences). The gap open penalty (default 10.0) is the score taken away when a gap is created. The default value assumes you are using the EBLOSUM62 matrix for protein sequences. The gap extension (default 0.5) penalty is added to the standard gap penalty for each base or residue in the gap. This is how long gaps are penalized.

As used herein, a polypeptide having an amino acid sequence having at least 80 % sequence identity to

the full-length sequence of a reference polypeptide sequence is to be understood as that the sequence has 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 91.50 %, 92.00 %, 92.50 %, 93.00 %, 93.50 %, 94.00 %, 94.50 %, 95.00 %, 95.50 %, 96.00 %, 96.50 %, 97.00 %, 97.50 %, 98.00 %, 98.50 %, 99.00 %, 99.50 %, 99.60 %, 99.70 %, 99.80 %, 99.90 %, 100 % sequence identity to the full-length of the amino acid sequence of the reference polypeptide sequence. Throughout the application, unless explicitly specified otherwise, a polypeptide (or DNA sequence) comprising/consisting/having an amino acid sequence (or nucleotide sequence) having at least 80% sequence identity to the full-length amino acid sequence (or nucleotide sequence) of a reference polypeptide (or nucleotide sequence), usually indicated with a SEQ ID NO or UniProt ID or Genbank NO., preferably has at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, more preferably has at least 85%, even more preferably has at least 90%, most preferably has at least 95%, sequence identity to the full length reference sequence.

For the purposes of this invention, percent identity is determined using MatGAT2.01 (Campanella *et al.*, 2003, BMC Bioinformatics 4:29). The following default parameters for protein are employed: (1) Gap cost Existence: 12 and Extension: 2; (2) The Matrix employed was BLOSUM65. In a preferred embodiment, sequence identity is calculated based on the full-length sequence of a given SEQ ID NO, i.e. the reference sequence, or a part thereof. Part thereof preferably means at least 50%, 60%, 70%, 80%, 90% or 95% of the complete reference sequence.

The term “cultivation” refers to the culture medium wherein the cell is cultivated or fermented, the cell itself, and the oligosaccharides that are produced by the cell in whole broth, i.e. inside (intracellularly) as well as outside (extracellularly) of the cell.

The terms “membrane transporter proteins” and “membrane proteins” are used interchangeably and refer to proteins that are part of or interact with the cell membrane and control the flow of molecules and information across the cell. The membrane proteins are thus involved in transport, be it import into or export out of the cell.

Such membrane transporter proteins can be porters, P-P-bond-hydrolysis-driven transporters, β -Barrel Porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators as defined by the Transporter Classification Database that is operated and curated by the Saier Lab Bioinformatics Group available via www.tcdb.org and providing a functional and phylogenetic classification of membrane transport proteins. This Transporter Classification Database details a comprehensive IUBMB approved classification system for membrane transporter proteins known as the Transporter Classification (TC) system. The TCDB classification searches as described here are defined based on TCDB. org as released on 17th June 2019.

Porters is the collective name of uniporters, symporters, and antiporters that utilize a carrier-mediated process (Saier *et al.*, Nucleic Acids Res. 44 (2016) D372-D379). They belong to the electrochemical potential-driven transporters and are also known as secondary carrier-type facilitators. Membrane transporter proteins are included in this class when they utilize a carrier-mediated process to catalyse

uniport when a single species is transported either by facilitated diffusion or in a membrane potential-dependent process if the solute is charged; antiport when two or more species are transported in opposite directions in a tightly coupled process, not coupled to a direct form of energy other than chemiosmotic energy; and/or symport when two or more species are transported together in the same direction in a tightly coupled process, not coupled to a direct form of energy other than chemiosmotic energy, of secondary carriers (Forrest *et al.*, Biochim. Biophys. Acta 1807 (2011) 167-188). These systems are usually stereospecific. Solute:solute countertransport is a characteristic feature of secondary carriers. The dynamic association of porters and enzymes creates functional membrane transport metabolons that channel substrates typically obtained from the extracellular compartment directly into their cellular metabolism (Moraes and Reithmeier, Biochim. Biophys. Acta 1818 (2012), 2687-2706). Solutes that are transported via this porter system include but are not limited to cations, organic anions, inorganic anions, nucleosides, amino acids, polyols, phosphorylated glycolytic intermediates, osmolytes, siderophores.

Membrane transporter proteins are included in the class of P-P-bond hydrolysis-driven transporters if they hydrolyse the diphosphate bond of inorganic pyrophosphate, ATP, or another nucleoside triphosphate, to drive the active uptake and/or extrusion of a solute or solutes (Saier *et al.*, Nucleic Acids Res. 44 (2016) D372-D379). The membrane transporter protein may or may not be transiently phosphorylated, but the substrate is not phosphorylated. Substrates that are transported via the class of P-P-bond hydrolysis-driven transporters include but are not limited to cations, heavy metals, beta-glucan, UDP-glucose, lipopolysaccharides, teichoic acid.

The β -Barrel porins membrane transporter proteins form transmembrane pores that usually allow the energy independent passage of solutes across a membrane. The transmembrane portions of these proteins consist exclusively of β -strands which form a β -barrel (Saier *et al.*, Nucleic Acids Res. 44 (2016) D372-D379). These porin-type proteins are found in the outer membranes of Gram-negative bacteria, mitochondria, plastids, and possibly acid-fast Gram-positive bacteria. Solutes that are transported via these β -Barrel porins include but are not limited to nucleosides, raffinose, glucose, beta-glucosides, oligosaccharides.

The auxiliary transport proteins are defined to be proteins that facilitate transport across one or more biological membranes but do not themselves participate directly in transport. These membrane transporter proteins always function in conjunction with one or more established transport systems such as but not limited to outer membrane factors (OMFs), polysaccharide (PST) porters, the ATP-binding cassette (ABC)-type transporters. They may provide a function connected with energy coupling to transport, play a structural role in complex formation, serve a biogenic or stability function or function in regulation (Saier *et al.*, Nucleic Acids Res. 44 (2016) D372-D379). Examples of auxiliary transport proteins include but are not limited to the polysaccharide copolymerase family involved in polysaccharide transport, the membrane fusion protein family involved in bacteriocin and chemical toxin transport.

Putative transport protein comprises families which will either be classified elsewhere when the transport

function of a member becomes established or will be eliminated from the Transporter Classification system if the proposed transport function is disproven. These families include a member or members for which a transport function has been suggested, but evidence for such a function is not yet compelling (Saier et al., *Nucleic Acids Res.* 44 (2016) D372-D379). Examples of putative transporters classified in this group under the TCDB system as released on 17th June 2019 include but are not limited to copper transporters.

The phosphotransfer-driven group translocators are also known as the PEP-dependent phosphoryl transfer-driven group translocators of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS). The product of the reaction, derived from extracellular sugar, is a cytoplasmic sugar-phosphate. The enzymatic constituents, catalysing sugar phosphorylation, are superimposed on the transport process in a tightly coupled process. The PTS system is involved in many different aspects comprising in regulation and chemotaxis, biofilm formation, and pathogenesis (Lengeler, J. *Mol. Microbiol. Biotechnol.* 25 (2015) 79-93; Saier, J. *Mol. Microbiol. Biotechnol.* 25 (2015) 73-78). Membrane transporter protein families classified within the phosphotransfer-driven group translocators under the TCDB system as released on 17th June 2019 include PTS systems linked to transport of glucose-glucosides, fructose-mannitol, lactose-N,N'-diacetylchitobiose-beta-glucoside, glucitol, galactitol, mannose-fructose-sorbose and ascorbate.

The major facilitator superfamily (MFS) is a superfamily of membrane transporter proteins catalysing uniport, solute:cation (H⁺, but seldom Na⁺) symport and/or solute:H⁺ or solute:solute antiport. Most are of 400-600 amino acid residues in length and possess either 12, 14, or occasionally, 24 transmembrane α -helical spanners (TMSs) as defined by the Transporter Classification Database operated by the Saier Lab Bioinformatics Group (www.tcdb.org).

"SET" or "Sugar Efflux Transporter" as used herein refers to membrane proteins of the SET family which are proteins with InterPRO domain IPR004750 and/or are proteins that belong to the eggNOGv4.5 family ENOG410XTE9. Identification of the InterPro domain can be done by using the online tool on <https://www.ebi.ac.uk/interpro/> or a standalone version of InterProScan (<https://www.ebi.ac.uk/interpro/download.html>) using the default values. Identification of the orthology family in eggNOGv4.5 can be done using the online version or a standalone version of eggNOG-mapperv1 (<http://eggnogdb.embl.de/#/app/home>).

The term "Siderophore" as used herein is referring to the secondary metabolite of various microorganisms which are mainly ferric ion specific chelators. These molecules have been classified as catecholate, hydroxamate, carboxylate and mixed types. Siderophores are in general synthesized by a nonribosomal peptide synthetase (NRPS) dependent pathway or an NRPS independent pathway (NIS). The most important precursor in NRPS-dependent siderophore biosynthetic pathway is chorismate. 2, 3-DHBA could be formed from chorismate by a three-step reaction catalysed by isochorismate synthase, isochorismatase, and 2, 3-dihydroxybenzoate-2, 3-dehydrogenase. Siderophores can also be formed from

salicylate which is formed from isochorismate by isochorismate pyruvate lyase. When ornithine is used as precursor for siderophores, biosynthesis depends on the hydroxylation of ornithine catalysed by L-ornithine N5-monooxygenase. In the NIS pathway, an important step in siderophore biosynthesis is N(6)-hydroxylysine synthase.

- 5 A transporter is needed to export the siderophore outside the cell. Four superfamilies of membrane proteins are identified so far in this process: the major facilitator superfamily (MFS); the Multidrug/Oligosaccharidyl-lipid/Polysaccharide Flippase Superfamily (MOP); the resistance, nodulation and cell division superfamily (RND); and the ABC superfamily. In general, the genes involved in siderophore export are clustered together with the siderophore biosynthesis genes. The term
10 "siderophore exporter" as used herein refers to such transporters needed to export the siderophore outside of the cell.

The ATP-binding cassette (ABC) superfamily contains both uptake and efflux transport systems, and the members of these two groups generally cluster loosely together. ATP hydrolysis without protein phosphorylation energizes transport. There are dozens of families within the ABC superfamily, and family
15 generally correlates with substrate specificity. Members are classified according to class 3.A.1 as defined by the Transporter Classification Database operated by the Saier Lab Bioinformatics Group available via www.tcdb.org and providing a functional and phylogenetic classification of membrane transporter proteins.

The term "enabled efflux" means to introduce the activity of transport of a solute over the cytoplasm
20 membrane and/or the cell wall. Said transport may be enabled by introducing and/or increasing the expression of a transporter protein as described in the present invention. The term "enhanced efflux" means to improve the activity of transport of a solute over the cytoplasm membrane and/or the cell wall. Transport of a solute over the cytoplasm membrane and/or cell wall may be enhanced by introducing and/or increasing the expression of a membrane transporter protein as described in the present
25 invention. "Expression" of a membrane transporter protein is defined as "overexpression" of the gene encoding said membrane transporter protein in the case said gene is an endogenous gene or "expression" in the case the gene encoding said membrane transporter protein is a heterologous gene that is not present in the wild type strain or cell.

The terms "precursor" as used herein refers to substances which are taken up or synthesized by the cell
30 for the specific production of an oligosaccharide according to the present invention. In this sense a precursor can be an acceptor as defined herein, but can also be another substance, metabolite, which is first modified within the cell as part of the biochemical synthesis route of the oligosaccharide. Examples of such precursors comprise the acceptors as defined herein, and/or glucose, galactose, fructose, glycerol, sialic acid, fucose, mannose, maltose, sucrose, lactose, dihydroxyacetone, glucosamine, N-acetyl-
35 glucosamine, mannosamine, N-acetyl-mannosamine, galactosamine, N-acetyl-galactosamine, phosphorylated sugars like e.g. but not limited to glucose-1-phosphate, galactose-1-phosphate, glucose-

6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, mannose-6-phosphate, mannose-1-phosphate, glycerol-3-phosphate, glyceraldehyde-3-phosphate, dihydroxyacetone-phosphate, glucosamine-6-phosphate, N-acetyl-glucosamine-6-phosphate, N-acetylmannosamine-6-phosphate, N-acetylglucosamine-1-phosphate, N-acetyl-neuraminic acid-9-phosphate and/or nucleotide-activated sugars as defined herein like e.g. UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, CMP-sialic acid, GDP-mannose, GDP-4-dehydro-6-deoxy- α -D-mannose, GDP-fucose. Optionally, the cell is transformed to comprise at least one nucleic acid sequence encoding a protein selected from the group consisting of lactose transporter, N-acetylneuraminic acid transporter, fucose transporter, transporter for a nucleotide-activated sugar wherein said transporter internalizes a to the medium added precursor for oligosaccharide synthesis

The term "acceptor" as used herein refers to di- or oligosaccharides which can be modified by a glycosyltransferase. Examples of such acceptors comprise lactose, lacto-N-biose (LNB), lacto-N-triose, lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), N-acetyl-lactosamine (LacNAc), lacto-N-pentaose (LNP), lacto-N-neopentaose, para lacto-N-pentaose, para lacto-N-neopentaose, lacto-N-novopentaose I, lacto-N-hexaose (LNH), lacto-N-neohexaose (LNnH), para lacto-N-neohexaose (pLNnH), para lacto-N-hexaose (pLNH), lacto-N-heptaose, lacto-N-neoheptaose, para lacto-N-neoheptaose, para lacto-N-heptaose, lacto-N-octaose (LNO), lacto-N-neooctaose, iso lacto-N-octaose, para lacto-N-octaose, iso lacto-N-neooctaose, novo lacto-N-neooctaose, para lacto-N-neooctaose, iso lacto-N-nonaose, novo lacto-N-nonaose, lacto-N-nonaose, lacto-N-decaose, iso lacto-N-decaose, novo lacto-N-decaose, lacto-N-neodecaose, galactosyllactose, a lactose extended with 1, 2, 3, 4, 5, or a multiple of N-acetyllactosamine units and/or 1, 2, 3, 4, 5, or a multiple of, Lacto-N-biose units, and oligosaccharide containing 1 or multiple N-acetyllactosamine units and or 1 or multiple lacto-N-biose units or an intermediate into oligosaccharide, fucosylated and sialylated versions thereof.

Throughout the application, unless explicitly stated otherwise, the features "synthesize", "synthesized" and "synthesis" are interchangeably used with the features "produce", "produced" and "production", respectively.

Detailed description of the invention

According to a first aspect, the present invention provides a metabolically engineered cell for the production of a mixture comprising at least three different mammalian milk oligosaccharides (MMOs), preferably lactose-based MMOs, more preferably human milk oligosaccharides (HMOs), i.e. a cell which is metabolically engineered for the production of a mixture comprising at least three different MMOs, preferably HMOs. Throughout the application, unless explicitly stated otherwise, the feature "mammalian milk oligosaccharide" or "mammalian milk oligosaccharides" or "MMO" or "MMOs" is preferably replaced with "lactose-based mammalian milk oligosaccharide" or "lactose-based mammalian milk oligosaccharides" or "lactose-based MMO" or "lactose-based MMOs", respectively, more preferably

replaced with “human milk oligosaccharide” or “human milk oligosaccharides” or “HMO” or “HMOs”, respectively. Herein, a single metabolically engineered cell is provided which is capable to express, preferably expresses, at least two glycosyltransferases and is capable of synthesizing one or more sugar-nucleotide(s) which is/are donor(s) for said glycosyltransferases. Throughout the application, unless explicitly stated otherwise, a “genetically modified cell” or “metabolically engineered cell” preferably means a cell which is genetically modified or metabolically engineered, respectively, for the production of said mixture comprising at least three different MMOs according to the invention. In the context of the invention, the at least three different MMOs of said mixture as disclosed herein preferably do not occur in the wild type progenitor of said metabolically engineered cell.

According to second aspect, the present invention provides a method for the production of a mixture comprising at least three different mammalian milk oligosaccharides. The method comprises the steps of:

- i) providing a cell, preferably a single cell, that is capable to express, preferably expresses, at least two glycosyltransferases and is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases, and
- ii) cultivating said cell under conditions permissive to express said glycosyltransferases and to synthesize said nucleotide-sugar(s),
- iii) preferably, separating at least one of said mammalian milk oligosaccharides from the cultivation, more preferably separating all of said mammalian milk oligosaccharides from said cultivation.

In the scope of the present invention, permissive conditions are understood to be conditions relating to physical or chemical parameters including but not limited to temperature, pH, pressure, osmotic pressure and product/precursor/acceptor concentration.

In a particular embodiment, the permissive conditions may include a temperature-range of 30 +/- 20 degrees centigrade, a pH-range of 7 +/- 3.

In the context of the invention, it should be understood that said cell produces said oligosaccharides intracellularly. The skilled person will further understand that a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside the cell via passive or active transport.

According to the invention, said method for the production of a mixture comprising at least three different MMOs can make use of a non-metabolically engineered cell or can make use of a metabolically engineered cell, i.e. a cell which is metabolically engineered for the production of said mixture comprising at least three different MMOs.

According to a preferred embodiment of the method and cell according to the invention, the metabolically engineered cell is modified with gene expression modules wherein the expression from any one of said expression modules is constitutive or is created by a natural inducer.

Said expression modules are also known as transcriptional units and comprise polynucleotides for expression of recombinant genes including coding gene sequences and appropriate transcriptional and/or translational control signals that are operably linked to the coding genes. Said control signals comprise promoter sequences, untranslated regions, ribosome binding sites, terminator sequences. Said expression modules can contain elements for expression of one single recombinant gene but can also contain elements for expression of more recombinant genes or can be organized in an operon structure for integrated expression of two or more recombinant genes. Said polynucleotides may be produced by recombinant DNA technology using techniques well-known in the art. Methods which are well known to those skilled in the art to construct expression modules include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al. (2001) *Molecular Cloning: a laboratory manual*, 3rd Edition, Cold Spring Harbor Laboratory Press, CSH, New York or to *Current Protocols in Molecular Biology*, John Wiley and Sons, N.Y. (1989 and yearly updates).

According to a preferred embodiment of the present invention, the cell is modified with one or more expression modules. The expression modules can be integrated in the genome of said cell or can be presented to said cell on a vector. Said vector can be present in the form of a plasmid, cosmid, phage, liposome, or virus, which is to be stably transformed/transfected into said metabolically engineered cell. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. These vectors may contain selection markers such as but not limited to antibiotic markers, auxotrophic markers, toxin-antitoxin markers, RNA sense/antisense markers. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., see above. For recombinant production, cells can be genetically engineered to incorporate expression systems or portions thereof or polynucleotides of the invention. Introduction of a polynucleotide into the cell can be effected by methods described in many standard laboratory manuals, such as Davis et al., *Basic Methods in Molecular Biology*, (1986), and Sambrook et al., 1989, *supra*.

As used herein an expression module comprises polynucleotides for expression of at least one recombinant gene. Said recombinant gene is involved in the expression of a polypeptide acting in the synthesis of said MMO mixture; or said recombinant gene is linked to other pathways in said host cell that are not involved in the synthesis of said mixture of three or more MMOs. Said recombinant genes encode

endogenous proteins with a modified expression or activity, preferably said endogenous proteins are overexpressed; or said recombinant genes encode heterologous proteins that are heterogeneously introduced and expressed in said modified cell, preferably overexpressed. The endogenous proteins can have a modified expression in the cell which also expresses a heterologous protein.

- 5 According to a preferred embodiment of the present invention, the expression of each of said expression modules is constitutive or created by a natural inducer. As used herein, constitutive expression should be understood as expression of a gene that is transcribed continuously in an organism. Expression that is created by a natural inducer should be understood as a facultative or regulatory expression of a gene that is only expressed upon a certain natural condition of the host (e.g. organism being in labor, or during
- 10 lactation), as a response to an environmental change (e.g. including but not limited to hormone, heat, cold, light, oxidative or osmotic stress / signaling), or dependent on the position of the developmental stage or the cell cycle of said host cell including but not limited to apoptosis and autophagy.

The present invention provides different types of cells for said production of a mammalian milk oligosaccharide mixture comprising three or more MMOs with a single metabolically engineered cell. For

15 example, the present invention provides a cell wherein said cell expresses two different glycosyltransferases and said cell synthesizes one single nucleotide-sugar which is donor for both said expressed glycosyltransferases. The present invention also provides a cell wherein said cell expresses three different glycosyltransferases and said cell synthesizes one single nucleotide-sugar which is donor for all of said three expressed glycosyltransferases. The present invention also provides a cell wherein said

20 cell expresses two different glycosyltransferases and said cell synthesizes two different nucleotide-sugars whereby a first nucleotide-sugar is donor for the first glycosyltransferase and a second nucleotide-sugar is donor for the second glycosyltransferase. The present invention also provides a cell wherein said cell expresses three or more glycosyltransferases and said cell synthesizes one or more different nucleotide-sugar(s) which is/are donor(s) for all of said expressed glycosyltransferases.

- 25 In the method and cell described herein, the cell preferably comprises multiple copies of the same coding DNA sequence encoding for one protein. In the context of the present invention, said protein can be a glycosyltransferase, a membrane protein or any other protein as disclosed herein. Throughout the application, the feature "multiple" means at least 2, preferably at least 3, more preferably at least 4, even more preferably at least 5.

30 In an embodiment of the method and/or cell according to the invention, said mixture comprises at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different mammalian milk oligosaccharides. In a preferred embodiment, any one of said mammalian milk oligosaccharides is a human milk oligosaccharide, preferably all said oligosaccharides are human milk oligosaccharides.

- 35 In an optional embodiment of the method and/or cell according to the invention, the mixture according to the invention further comprises LacdiNAc (i.e. GalNAc-b1,4-GICNAc) and/or GalNAc-b1,4-glucose.

In an additional and/or alternative embodiment of the method and/or cell according to the invention, the mixture comprises at least three different mammalian milk oligosaccharides differing in degree of polymerization (DP). The degree of polymerization of an oligosaccharide refers to the number of monosaccharide units present in the oligosaccharide structure. As used herein, the degree of polymerization of an oligosaccharide is three (DP3) or more, the latter comprising any one of 4 (DP4), 5 (DP5), 6 (DP6) or longer. The mammalian milk oligosaccharide mixture as described herein preferably comprises at least three different mammalian milk oligosaccharides wherein all mammalian milk oligosaccharides present in the mixture have a different degree of polymerization from each other. For example, said oligosaccharide mixture consists of three MMOs, wherein the first MMO is a trisaccharide with a degree of polymerization of 3 (DP3), the second MMO is a tetrasaccharide with a degree of polymerization of 4 (DP4) and the third MMO is a pentasaccharide with a degree of polymerization of 5 (DP5). According to one embodiment of the method and/or cell of the invention, the cell produces a mixture comprising four different MMOs or more than four different MMOs. In one embodiment, such mixture comprises at least four different MMOs wherein three of the oligosaccharides have a different degree of polymerization. In one embodiment, all of said MMOs in the mixture have a different degree of polymerization as described herein.

In an additional and/or alternative preferred embodiment of the method and/or cell according to the invention, the MMO mixture as described herein is composed of charged and neutral mammalian milk oligosaccharides. As such, the present invention preferably provides a cell which is metabolically engineered for the production of a mixture comprising at least three different MMOs and wherein said mixture is composed of charged and neutral MMOs, wherein said cell is capable to express at least two glycosyltransferases, preferably at least three, more preferably at least four, even more preferably at least five, most preferably at least six, at least seven, at least eight glycosyltransferases, and is capable to synthesize one or more nucleotide-sugar(s), preferably at least two, more preferably at least three, even more preferably at least four, most preferably at least five nucleotide-sugars, wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases. In other words, the present invention preferably provides a metabolically engineered cell for the production of, preferably said cell producing, a mixture comprising at least three different MMOs and wherein said mixture is composed of charged and neutral MMOs, wherein said cell is genetically modified for the production of said mixture, is capable to express at least two glycosyltransferases, preferably at least three, more preferably at least four, even more preferably at least five, most preferably at least six, at least seven, at least eight glycosyltransferases, and is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases. Further, the present invention preferably provides a method for the production of a mixture comprising at least three different MMOs and wherein said mixture is composed of charged and neutral MMOs, the method comprises the steps as described herein (wherein it is

preferred to provide a metabolically engineered cell as disclosed herein). Charged oligosaccharides (MMOs) are oligosaccharide structures that contain one or more negatively charged monosaccharide subunits including N-acetylneuraminic acid (Neu5Ac), commonly known as sialic acid, N-glycolylneuraminic acid (Neu5Gc), Neu4Ac, Neu5Ac9N₃, Neu4,5Ac₂, Neu5,7Ac₂, Neu5,9Ac₂, Neu5,7(8,9)Ac₂, glucuronate and galacturonate. Charged oligosaccharides (MMOs) are also referred to as acidic oligosaccharides (MMOs). Throughout the application, said charged MMOs are preferably sialylated MMOs. Sialic acid belongs to the family of derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulonic acid). Neu5Gc is a derivative of sialic acid, which is formed by hydroxylation of the N-acetyl group at C5 of Neu5Ac. Neutral oligosaccharides (MMOs) are non-sialylated oligosaccharides (MMOs), and thus do not contain an acidic monosaccharide subunit. Neutral oligosaccharides (MMOs) comprise non-charged fucosylated oligosaccharides (MMOs) that contain one or more fucose subunits in their glycan structure as well as non-charged non-fucosylated oligosaccharides (MMOs) that lack any fucose subunit.

According to an additional and/or alternative embodiment of the method and/or cell of the invention, at least one of said oligosaccharides of said mixture is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylglactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

Preferably, the MMO mixture comprises at least one fucosylated oligosaccharide as defined herein.

Alternatively or additionally, the MMO mixture comprises at least one sialylated oligosaccharide as defined herein.

Alternatively or additionally, the mixture of MMOs comprises at least one MMO of three or more monosaccharide subunits linked to each other via glycosidic bonds, wherein at least one of said monosaccharide residues is a N-acetylglucosamine (GlcNAc) residue. Said oligosaccharide can contain more than one GlcNAc residue, e.g. two, three or more. Said oligosaccharide can be a neutral oligosaccharide or a charged oligosaccharide, e.g. also comprising sialic acid structures. GlcNAc can be present at the reducing end of the oligosaccharide. Said GlcNAc can also be present at the non-reducing end of said oligosaccharide. Said GlcNAc can also be present within the oligosaccharide structure. GlcNAc can be linked to other monosaccharide subunits comprising galactose, fucose, Neu5Ac, Neu5Gc, glucose.

Alternatively or additionally, the MMO mixture comprises at least one galactosylated MMO and contains at least one galactose monosaccharide subunit. Said galactosylated oligosaccharide is a saccharide structure comprising at least three monosaccharide subunits linked to each other via glycosidic bonds, wherein at least one of said monosaccharide subunit is a galactose. Said galactosylated oligosaccharide can contain more than one galactose residue, e.g. two, three or more. Said galactosylated oligosaccharide can be a neutral oligosaccharide or a charged oligosaccharide, e.g. also comprising sialic acid structures. Galactose can be linked to other monosaccharide subunits comprising glucose, GlcNAc, fucose, sialic acid.

In an additional and/or alternative preferred embodiment of the method and/or cell according to the invention, the MMO mixture as described herein is composed of charged (preferably sialylated) and neutral MMOs, wherein the relative abundance of said charged (preferably sialylated) MMOs in said mixture is at least 5%, preferably at least 7%, more preferably at least 10%. Preferably, the relative abundance of said charged MMOs in said mixture is less than 20%, preferably less than 15%. As such, the relative abundance of said charged MMOs in said mixture is preferably 5-20%, preferably 5-15%, more preferably 10-15%, even more preferably 12-14%, most preferably reflecting the relative abundance of charged MMOs in human breast milk and/or colostrum. The skilled person will further understand that if the relative abundance of the charged oligosaccharides in the mixture is defined, inevitably the remainder fraction of oligosaccharides in the mixture are neutral oligosaccharides.

In a preferred embodiment of the method and/or cell according to the invention, said neutral MMOs do not comprise non-fucosylated MMOs. In an alternative preferred embodiment, said neutral MMOs do not comprise fucosylated MMOs. In a more preferred embodiment, said neutral MMOs comprise fucosylated MMO(s) and non-fucosylated MMO(s). In an even more preferred embodiment, the relative abundance of fucosylated MMOs in the neutral MMOs fraction of said mixture is at least 10%, preferably at least 20%, more preferably at least 30%, most preferably at least 35%. Preferably, the relative abundance of fucosylated MMOs in the neutral MMOs fraction of said mixture is 10-60%, preferably 20-60%, more preferably 30-60%, even more preferably 30-50%, most preferably reflecting the relative abundance of fucosylated MMOs in the neutral MMOs fraction in human breast milk and/or colostrum.

In an additional and/or alternative embodiment of the method and/or cell according to the invention, the MMO mixture as described herein comprises fucosylated MMO(s) with a relative abundance in said mixture is at least 10%, preferably at least 20%, more preferably at least 30%, even more preferably at least 40%, even more preferably at least 50%, most preferably at least 55%. Preferably, the relative abundance of said fucosylated MMOs in said mixture is less than 90%, preferably less than 80%, more preferably less than 70%, even more preferably less than 60%. As such, the relative abundance of said fucosylated MMOs in said mixture is preferably 10-90%, preferably 20-80%, more preferably 30-60%, even more preferably 40-55%, most preferably reflecting the relative abundance of fucosylated MMOs in human breast milk and/or colostrum.

As such, in an additional and/or alternative embodiment of the method and/or cell according to the invention, the MMO mixture as described herein is composed of charged (preferably sialylated) and neutral MMOs, wherein the relative abundance of charged (preferably sialylated) MMOs and/or fucosylated MMOs is as described herein.

In an additional and/or alternative embodiment of the method and/or cell according to the invention, the relative abundance of each oligosaccharide in the mixture as described herein is at least 5%, preferably at least 10%.

In the context of this invention, said oligosaccharide mixture as disclosed herein is preferably the direct

result of metabolically engineering a cell as described herein. This means that preferably at least one, more preferably at least two, even more preferably at least three, most preferably all, of the oligosaccharides in the mixture according to the invention are not produced by the wild type progenitor of said metabolically engineered cell.

5 In the context of the present invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can comprise lactose-based MMOs, LNB-based MMOs, LacNAc-based MMOs as described herein and other oligosaccharides that are not MMOs. In a preferred embodiment of the method and/or cell according to the invention, said mixture comprises at least three MMOs, preferably lactose-based MMOs, more preferably HMOs, and optionally at least one, preferably
10 at least two, more preferably at least three, even more preferably at least four, other oligosaccharides that are not MMOs. In an alternative and/or additional preferred embodiment of the method and/or cell according to the invention, mammalian milk oligosaccharides constitute at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, of the oligosaccharide mixture according to the invention. In a more preferred embodiment of the method
15 and/or cell according to the invention, all the oligosaccharides in said mixture are MMOs, preferably lactose-based MMOs, more preferably HMOs. As already stated herein, it is preferred that the mixture as disclosed herein is the direct result of metabolically engineering a cell as described herein.

The names of the MMOs as described herein are in accordance with the oligosaccharide names and formulae as published by Urashima et al. (Trends in Glycoscience and Glycotechnology, 2018, vol 30, no
20 72, pag SE51-SE65) and references therein and as published in "Prebiotics and Probiotics in human milk. Origins and Functions of Milk-Borne Oligosaccharides and Bacteria", Chapters 2 & 3, Eds M. McGuire, M. McGuire, L. Bode, Elsevier, Academic Press, pag 506).

Throughout the application, unless explicitly stated otherwise, the feature "at least one" is preferably replaced with "one", likewise the feature "at least two" is preferably replaced with "two", etc.

25 In a more preferred embodiment of the method and/or cell according to the invention, the MMO mixture comprising at least three different MMOs as described herein is composed of charged (preferably sialylated) and neutral MMOs, and wherein said mixture comprises, consists of, or consists essentially of:

a) at least one, preferably at least two, more preferably at least three, even more preferably at least
30 four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different charged MMOs preferably selected from:
a1) lactose-based charged MMOs, preferably any one of 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH
35 III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-
NO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH,

FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-b1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,6-[Gal-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, and/or a2) LNB-based charged MMOs, preferably any one of 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB); and/or

a3) LacNAc-based charged oligosaccharides, preferably any one of 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc; and

combined with

b) at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different neutral fucosylated MMOs preferably selected from:

b1) lactose-based neutral fucosylated MMOs, preferably any one of 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH; and/or

b2) LNB-based neutral fucosylated MMOs, preferably any one of 2'FLNB, 4'FLNB; and/or

Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc combined with at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different neutral fucosylated MMOs preferably selected from 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc and/or combined with at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different neutral non-fucosylated MMOs preferably selected from Gal-a1,4-Gal-b1,4-Glc (Gal-a1,4-lactose or globotriose), β 3'-galactosyllactose, β 6'-galactosyllactose, Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), LNnH, LNnH, iso-LNO, novo-LNO, novo-LNnO, LND, iso-LND, GalNAc-a1,3-Gal-b1,4-Glc, novo-LNP I, Gal-novo-LNP I, Gal-novo-LNP II, Gal-novo-LNP III, iso-LNT, DGalLNnH, galilipentasaccharide, Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, novo-LNT, poly-LacNAc, LacDiNAc, optionally combined with any one or more of charged and/or neutral oligosaccharides that are not MMOs.

More preferred mixtures in this context of the invention comprise mixtures of at least three different charged and neutral MMOs wherein said mixtures comprise, consist of or consist essentially of at least three charged and neutral MMOs chosen from the list comprising 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-LNO II,

DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH
I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH
sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc,
Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-
Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-
Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-
a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-
a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-
GlcNAc-b1,6-[Gal-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-
[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-
GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH,
Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose
(6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-
GlcNAc, 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LND FH I, LND FH II, LND FH III, F-LNH
I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-
LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-
para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-
LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-
iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-
para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-
LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND
III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND
VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-
tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-
b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-
heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF
DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc, Gal-a1,4-Gal-
b1,4-Glc (Gal-a1,4-lactose or globotriose), β3'-galactosyllactose, β6'-galactosyllactose, Lacto-N-triose II
(LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), LN H, LNnH, iso-LNO, novo-LNO, novo-LNnO,
LND, iso-LND, GalNAc-a1,3-Gal-b1,4-Glc, novo-LNP I, Gal-novo-LNP I, Gal-novo-LNP II, Gal-novo-LNP III,
iso-LNT, DGalLNnH, galilipentasaccharide, Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-
Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-
b1,3-Gal-b1,4-Glc, novo-LNT, poly-LacNAc and LacDiNAc.

35 An example of said preferred mixtures comprises a mixture comprising, consisting of or consisting essentially of 3'-sialyllactose, 6'-sialyllactose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, 2'-fucosyllactose (2'FL), 3-fucosyllactose (3-FL), difucosyllactose, Lacto-N-fucopentaose I (LNFP-I; Fuc-a1,2-

Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc), Lacto-N-fucopentaose II (LNFP-II; Gal-b1,3-[Fuc-a1,4]-GlcNAc-b1,3-Gal-b1,4-Glc), Lacto-N-fucopentaose V (LNFP-V; Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), Lacto-N-triose II (LN3) and Lacto-N-tetraose (LNT).

Another example of said preferred mixtures comprises a mixture comprising, consisting of or consisting essentially of 3'-sialyllactose, 6'-sialyllactose, sialylated lacto-N-triose, sialyllacto-N-neotetraose, Lacto-N-fucopentaose III (LNFP III; Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-Glc), Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT) and GalNAc-b1,3-LNnT.

Another example of said preferred mixtures comprises a mixture comprising, consisting of or consisting essentially of 3'-sialyllactose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, 3-fucosyllactose (3-FL), Lacto-N-fucopentaose V (LNFP-V; Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), Lacto-N-triose II (LN3) and Lacto-N-tetraose (LNT).

Another example of said preferred mixtures comprises a mixture comprising, consisting of or consisting essentially of 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, LN3, LNT and LNnT.

Another example of said preferred mixtures comprises a mixture comprising, consisting of or consisting essentially of 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, LN3, sialylated LN3, LNT, LNnT, sialylated LNT, fucosylated LNT, sialylated LNnT, fucosylated LNnT.

Exemplary mixtures in this context of the invention are described in the Examples section.

Other preferred mixtures in this context of the invention comprise mixtures of at least three charged and neutral fucosylated MMOS and no neutral non-fucosylated MMOs wherein said mixtures comprise, consist or consist essentially of at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different charged MMOs preferably selected from any one of 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-LNO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-b1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,6-[Gal-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-

Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc, combined with at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different neutral fucosylated MMOs preferably selected from any one of 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc, optionally combined with any one or more of charged and/or neutral oligosaccharides that are not MMOs.

More preferred mixtures in this context of the invention comprise mixtures of at least three different charged and neutral fucosylated MMOs and no neutral non-fucosylated MMOs wherein said mixtures comprise, consist of or consist essentially of at least three charged and neutral fucosylated MMOs chosen from the list comprising 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-LNO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-b1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,6-[Gal-b1,3]-Gal-

b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc, 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc.

Exemplary mixtures in this context of the invention are described in the Examples section.

Other preferred mixtures in this context of the invention comprise mixtures of at least three charged and neutral non-fucosylated MMOS and no neutral fucosylated MMOs wherein said mixtures comprise, consist or consist essentially of at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different charged MMOs preferably selected from any one of 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-NO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-

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More preferred mixtures in this context of the invention comprise mixtures of at least three different charged and neutral non-fucosylated MMOs and no neutral fucosylated MMOs wherein said mixtures comprise, consist of or consist essentially of at least three charged and neutral non-fucosylated MMOs chosen from the list comprising 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-LNO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,6-[Gal-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc, Gal-a1,4-Gal-b1,4-Glc (Gal-a1,4-lactose or globotriose), β 3'-galactosyllactose, β 6'-galactosyllactose, Lacto-N-triose

II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), LNH, LNnH, iso-LNO, novo-LNO, novo-LNnO, LND, iso-LND, GalNAc-a1,3-Gal-b1,4-Glc, novo-LNP I, Gal-novo-LNP I, Gal-novo-LNP II, Gal-novo-LNP III, iso-LNT, DGalLNnH, galilipentasaccharide, Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, novo-LNT, poly-LacNAc, LacDiNAc.

Exemplary mixtures in this context of the invention are described in the Examples section.

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More preferred mixtures in this context of the invention comprise mixtures of at least three different

neutral fucosylated and neutral non-fucosylated MMOs and no charged MMOs wherein said mixtures comprise, consist of or consist essentially of at least three neutral fucosylated and neutral non-fucosylated MMOs chosen from the list comprising 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-[Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3]-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc, Gal-a1,4-Gal-b1,4-Glc (Gal-a1,4-lactose or globotriose), β 3'-galactosyllactose, β 6'-galactosyllactose, Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), LNnH, LNnH, iso-LNO, novo-LNO, novo-LNnO, LND, iso-LND, GalNAc-a1,3-Gal-b1,4-Glc, novo-LNP I, Gal-novo-LNP I, Gal-novo-LNP II, Gal-novo-LNP III, iso-LNT, DGalLNnH, galilipentasaccharide, Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, novo-LNT, poly-LacNAc, LacDiNAc.

Exemplary mixtures in this context of the invention are described in the Examples section.

Other preferred mixtures in this context of the invention comprise mixtures of at least three charged MMOS and no neutral MMOs wherein said mixtures comprise, consist or consist essentially of at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different charged MMOs preferably selected from any one of 3'SL, 6'SL, F-SL, LSTa, LSTb, LSTc, LSTd, F-LSTa, F-LSTb, F-LSTc, S-LNH I, S-LNH II, S-LNnH I, S-LNnH II, S-para-LNnH, FS-LNH, FS-LNH I, FS-LNH II, FS-LNH III, FS-LNH IV, FS-LNnH I, FS-LNnH II, FS-para-LNnH I, FS-para-LNnH II, DFS-LNH I, DFS-LNH III, DFS-LNH IV, DFS-LNnH, S-LNO, FS-LNO I, FS-LNO II, FS-iso-LNO, DFS-iso-LNO I, DFS-iso-LNO II, DFS-LNO I, DFS-LNO II, DFS-LNO III, TFS-LNO, TFS-iso-LNO, DS-LNT, FDS-LNT I, FDS-LNT II, DS-LNH I, DS-LNH II, DS-LNnH, FDS-LNH I, FDS-LNH II, FDS-LNH III, FDS-LNnH, TS-LNH, SLNnD, FS-novo-LNP I, DF-para-LNH sulfate I, DF-para-LNH sulfate II, TF-para-LNH sulfate, Neu5Ac-a2,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-

a2,8-Neu5Ac-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,8-Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,6-[Neu5Ac-a2,3]-Gal-b1,4-Glc, Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Gal-b1,4-GlcNAc-b1,6-[Neu5Ac-a2,3-Gal-b1,3]-Gal-1,4-Glc, Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,6-[Gal-b1,3]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,4-Glc, Neu5Gc-a2,6-Gal-b1,4-Glc, Gal-b1,3-[Neu5Gc-a2,6]-Gal-b1,4-Glc, Neu5Gc-a2,3-Gal-b1,3-[Neu5Ac-a2,6]-Gal-b1,4-Glc, Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,6-[GlcNAc-b1,3]-Gal-b1,4-Glc, FS Gal-LNnH, DFSGal-LNnH, Gal-MSLNnH, MSDF-para-LNnH, Neu5GcLNnT, GM2 tetrasaccharide, SLNOa, 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc), 6'-sialyllactosamine (6'SLacNAc), Neu5Gc-a2,3-Gal-b1,4-GlcNAc.

Exemplary mixtures in this context of the invention are described in the Examples section.

Other preferred mixtures in this context of the invention comprise mixtures of at least three neutral MMOS and no charged MMOs wherein said mixtures comprise, consist or consist essentially of at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, at least eight, at least nine, at least ten different neutral MMOs preferably selected from any one of 2'FL, 3-FL, DiFL, LNFP-I, LNFP-II, LNFP-III, Gal-LNFP-III, LNFP-V, LNDFH I, LNDFH II, LNDFH III, F-LNH I, F-LNH II, F-LNH III, F-LNnH II, F-LNnH I, F-para-LNH I, F-para-LNH II, F-para-LNnH, DF-LNH II, DF-LNH I, DF-LNnH, DF-para-LNH, DF-para-LNH II, DF-para-LNH III, DF-para-LNnH, TF-LNH I, TF-LNH II, TF-para-LNH I, TF-para-LNH II, TF-para-LNnH, F-LNO I, F-LNO II, F-LNO III, F-LNnO, F-LNnO II, F-iso-LNO, F-iso-LNnO I, F-novo-LNnO, F-para-LNO, DF-iso-LNnO, DF-LNO I, DF-LNO II, DF-LNO III, DF-LNnO I, DF-LNnO II, DF-LNnO III, DF-iso-LNO I, DF-iso-LNO II, DF-iso-LNO III, DF-iso-LNO IV, DF-iso-LNO V, DF-iso-LNO VI, DF-iso-LNO VII, DF-para-LNnO, TF-LNO I, TF-LNO II, TF-LNnO, TF-iso-LNO I, TF-iso-LNO II, TF-iso-LNO III, TF-iso-LNO IV, TF-iso-LNnO, Tetra-F-iso-LNO, Tetra-F-para-LNO, Penta-F-iso-LNO, F-LND I, F-LND II, DF-LND I, DF-LND II, DF-LND III, DF-LND IV, DF-LND V, DF-LND VI, TriF-LND I, TriF-LND II, TriF-LND III, TriF-LND IV, TriF-LND V, TriF-LND VI, TriF-LND VII, TetraF-LND I, TetraF-LND II, TetraF-LND III, F-LNnD I, F-LNnD II, DF-LNnD, DF-novo-LND, A-tetrasaccharide, DF D Gal-LNnH (Gal-a1,3-Gal-b1,4-GlcNAc-b1,6-(Gal-a1,3-Gal-b1,4-(Fuc-a1,3)-GlcNAc-b1,3)-Gal-b1,4-Glc), 3-F-isoglobotriose, B-tetrasaccharide, B-pentasaccharide, B-hexasaccharide, B-heptasaccharide, DF DGal-LNnT (Gal-a1,3-Gal-b1,4-[Fuc-a1,3]-GlcNAc-b1,3-Gal-b1,4-[Fuc-a1,3]-Glc), TF DGal-LNnH a, TF DGal-LNnH b, DFGal-para-LNnH, 2'FLNB, 4'FLNB, 2'FLacNAc, 3-FLacNAc, Gal-a1,4-Gal-b1,4-Glc (Gal-a1,4-lactose or globotriose), β 3'-galactosyllactose, β 6'-galactosyllactose, Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), LNnH, LNnH, iso-LNO, novo-LNO, novo-LNnO, LND, iso-LND, GalNAc-a1,3-Gal-b1,4-Glc, novo-LNP I, Gal-novo-LNP I, Gal-novo-LNP II, Gal-novo-LNP III, iso-LNT, DGalLNnH, galilipentasaccharide, Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,3-Gal-b1,4-Glc, novo-LNT, poly-LacNAc, LacDiNAc.

An example of said preferred mixtures comprises a mixture comprising, consisting or consisting essentially of 2'-fucosyllactose (2'FL), Lacto-N-triose II (LN3), Lacto-N-tetraose (LNT) and Lacto-N-fucopentaose I

(LNFP-I; Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc).

Another example of said preferred mixtures comprises a mixture comprising, consisting or consisting essentially of Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), para-Lacto-N-neopentaose, para-Lacto-N-pentaose, para-Lacto-N-neohexaose and para-Lacto-N-hexaose.

5 Exemplary mixtures in this context of the invention are described in the Examples section.

For the production of lactose-based oligosaccharides as described herein, i.e. lactose-based mammalian milk oligosaccharides, and in an embodiment of the method and/or cell according to the invention, lactose can be added to the cultivation so that said cell can take it up passively or through active transport; or lactose can be produced by the cell (for example upon metabolically engineering the cell for this purpose
10 as known to the skilled person), preferably intracellularly. Lactose can hence be used as an acceptor in the synthesis of a mammalian milk oligosaccharide or human milk oligosaccharide, preferably all of the lactose-based MMOs or HMOs, which is/are preferably comprised in the oligosaccharide mixture according to the invention as described herein. A cell producing lactose can be obtained by expression of an N-acetylglucosamine beta-1,4-galactosyltransferase and an UDP-glucose 4-epimerase. More
15 preferably, the cell is modified for enhanced lactose production. Said modification can be any one or more chosen from the group comprising over-expression of an N-acetylglucosamine beta-1,4-galactosyltransferase, over-expression of an UDP-glucose 4-epimerase. Alternatively, a cell using lactose as acceptor in glycosylation reactions preferably has a transporter for the uptake of lactose from the cultivation. More preferably, the cell is optimized for lactose uptake. Said optimization can be over-
20 expression of a lactose transporter like a lactose permease from e.g. *E. coli*, *Kluyveromyces lactis* or *Lactobacillus casei* BL23. It is preferred to constitutively express the lactose permease. The lactose can be added at the start of the cultivation or it can be added as soon as enough biomass has been formed during the growth phase of the cultivation, i.e. the MMO production phase (initiated by the addition of lactose to the cultivation) is decoupled from the growth phase. In a preferred embodiment, the lactose is added
25 at the start and/or during the cultivation, i.e. the growth phase and production phase are not decoupled. In a preferred embodiment of the method and/or cell according to the invention, the cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s). With the term "lactose killing" is meant the hampered growth of the cell in medium in which lactose is present together with another carbon source. In a preferred embodiment, the
30 cell is genetically modified such that it retains at least 50% of the lactose influx without undergoing lactose killing, even at high lactose concentrations, as is described in WO 2016/075243. Said genetic modification comprises expression and/or over-expression of an exogenous and/or an endogenous lactose transporter gene by a heterologous promoter that does not lead to a lactose killing phenotype and/or modification of the codon usage of the lactose transporter to create an altered expression of said lactose transporter that
35 does not lead to a lactose killing phenotype. The content of WO 2016/075243 in this regard is incorporated by reference.

For the production of LNB-based oligosaccharides as described herein and in an additional and/or alternative embodiment of the method and/or cell according to the invention, LNB (i.e. lacto-N-biose, Gal-b1,3-GlcNAc) can be added to the cultivation so that said cell can take it up passively or through active transport; or LNB can be produced by the cell (for example upon metabolically engineering the cell for this purpose as known to the skilled person), preferably intracellularly. LNB can hence be used as an acceptor in the synthesis of a LNB-based oligosaccharide, preferably all of the LNB-based oligosaccharides, which is/are preferably comprised in the oligosaccharide mixture according to the invention as described herein. A cell producing LNB can be obtained by expression of an N-acetylglucosamine beta-1,3-galactosyltransferase which can modify GlcNAc (produced in the cell and/or taken up passively or through active transport) to form LNB. Preferably, a cell producing LNB is capable to express, preferably expresses, enzymes required for the synthesis of GlcNAc, such as glucosamine 6-phosphate N-acetyltransferase, phosphatase, N-acetylglucosamine beta-1,3-galactosyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, and UDP-glucose 4-epimerase, preferably a glucosamine 6-phosphate N-acetyltransferase and a phosphatase (preferably a HAD-like phosphatase). Preferably, the cell is metabolically engineered for production of LNB. More preferably, the cell is metabolically engineered for enhanced production of LNB. The cell is preferably modified to express and/or over-express any one or more of the polypeptides comprising glucosamine 6-phosphate N-acetyltransferase, phosphatase, N-acetylglucosamine beta-1,3-galactosyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, and UDP-glucose 4-epimerase.

A cell using LNB as acceptor in glycosylation reactions preferably has a transporter for the uptake of LNB from the cultivation. More preferably, the cell is optimized for LNB uptake. Said optimization can be over-expression of a LNB transporter like a lactose permease from e.g. *E. coli*, *Kluyveromyces lactis* or *Lactobacillus casei* BL23. It is preferred to constitutively express the lactose permease. The LNB can be added at the start of the cultivation or it can be added as soon as enough biomass has been formed during the growth phase of the cultivation, i.e. the oligosaccharide production phase (initiated by the addition of LNB to the cultivation) is decoupled from the growth phase. In a preferred embodiment, the LNB is added at the start and/or during the cultivation, i.e. the growth phase and production phase are not decoupled. For the production of LacNAc-based oligosaccharides as described herein and in an additional and/or alternative embodiment of the method and/or cell according to the invention, LacNAc (i.e. N-acetyllactosamine, Gal-b1,4-GlcNAc) can be added to the cultivation so that said cell can take it up passively or through active transport; or LacNAc can be produced by the cell (for example upon metabolically engineering the cell for this purpose as known to the skilled person), preferably intracellularly. LacNAc can hence be used as an acceptor in the synthesis of a LacNAc-based oligosaccharide, preferably all of the LacNAc-based oligosaccharides, which is/are preferably comprised in the oligosaccharide mixture according to the invention as described herein. A cell producing LacNAc can be obtained by expression of an N-acetylglucosamine beta-1,4-galactosyltransferase which can

modify GlcNAc (produced in the cell and/or taken up passively or through active transport) to form LacNAc. Preferably, a cell producing LacNAc is capable to express, preferably expresses, enzymes required for the synthesis of GlcNAc, such as glucosamine 6-phosphate N-acetyltransferase, phosphatase, N-acetylglucosamine beta-1,4-galactosyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, and UDP-glucose 4-epimerase, preferably a glucosamine 6-phosphate N-acetyltransferase and a phosphatase (preferably a HAD-like phosphatase). Preferably, the cell is metabolically engineered for production of LacNAc. More preferably, the cell is metabolically engineered for enhanced production of LacNAc. The cell is preferably modified to express and/or over-express any one or more of the polypeptides comprising glucosamine 6-phosphate N-acetyltransferase, phosphatase, N-acetylglucosamine beta-1,4-galactosyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, and UDP-glucose 4-epimerase.

A cell using LacNAc as acceptor in glycosylation reactions preferably has a transporter for the uptake of LacNAc from the cultivation. More preferably, the cell is optimized for LacNAc uptake. Said optimization can be over-expression of a LNB transporter like a lactose permease from e.g. *E. coli*, *Kluyveromyces lactis* or *Lactobacillus casei* BL23. It is preferred to constitutively express the lactose permease. The LacNAc can be added at the start of the cultivation or it can be added as soon as enough biomass has been formed during the growth phase of the cultivation, i.e. the oligosaccharide production phase (initiated by the addition of LacNAc to the cultivation) is decoupled from the growth phase. In a preferred embodiment, the LacNAc is added at the start and/or during the cultivation, i.e. the growth phase and production phase are not decoupled.

In an additional and/or alternative preferred embodiment of the method and/or cell according to the invention, said cell is capable to express at least two, preferably at least three, more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, glycosyltransferases as described herein.

In an additional and/or alternative preferred embodiment of the method and/or cell according to the invention, at least one, preferably at least two, more preferably all, of said glycosyltransferases are involved in the production of said mixture comprising at least three different mammalian milk oligosaccharides.

In an additional and/or alternative preferred embodiment of the method and/or cell according to the invention, any one of said glycosyltransferases is preferably chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases as defined herein.

In a preferred embodiment, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase. In another preferred embodiment, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase. In another preferred embodiment, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase. In another preferred embodiment, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase. In another preferred embodiment, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase. In another preferred embodiment, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase. In another preferred embodiment, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase.

In a further embodiment of the method and/or cell of the invention, the cell is modified in the expression or activity of at least one, preferably at least two, more preferably all, of said glycosyltransferases. In a preferred embodiment, said glycosyltransferase is an endogenous protein of the cell with a modified expression or activity, preferably said endogenous glycosyltransferase is overexpressed; alternatively said glycosyltransferase is an heterologous protein that is heterogeneously introduced and expressed in said cell, preferably overexpressed. Said endogenous glycosyltransferase can have a modified expression in the cell which also expresses a heterologous glycosyltransferase.

In another embodiment of the method and/or cell of the present invention, at least one, preferably at least two, of said glycosyltransferases is a fucosyltransferase and the cell is capable to synthesize GDP-Fuc. The GDP-fucose can be provided by an enzyme expressed in the cell or by the metabolism of the cell. Such cell producing GDP-fucose can express an enzyme converting, e.g., fucose, which is to be added to the cell, to GDP-fucose. This enzyme may be, e.g., a bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase, like Fkp from *Bacteroides fragilis*, or the combination of one separate fucose kinase together with one separate fucose-1-phosphate guanylyltransferase like they are known from several species including *Homo sapiens*, *Sus scrofa* and *Rattus norvegicus*. In a preferred embodiment of the method and/or cell of the present invention, said cell is capable of expressing at least one, preferably at least two, fucosyltransferase(s) selected from alpha-1,2-fucosyltransferases, alpha-1,3/1,4-fucosyltransferases and alpha-1,6-fucosyltransferases. Preferably, said fucosyltransferases are selected from organisms like e.g. *Helicobacter* species like e.g. *Helicobacter pylori*, *Helicobacter mustelae*, *Akkermansia* species like e.g. *Akkermansia muciniphila*, *Bacteroides* species like e.g. *Bacteroides fragilis*,

Bacteroides vulgatus, Bacteroides ovatus, E. coli species like e.g. E. coli O126, E. coli O55:H7, Lachnospiraceae species, Tannerella species, Clostridium species, Salmonella species like e.g. Salmonella enterica, Methanosphaerula palustries, Butyrivibrio species, Prevotella species, Porphyromonas species like e.g. Porphyromonas catoniae, Arabidopsis thaliana, Homo sapiens, Mus musculus. In a more preferred embodiment of the method and/or cell of the present invention, said fucosyltransferases are selected from the list comprising alpha-1,2-fucosyltransferases and alpha-1,3/1,4-fucosyltransferases.

Preferably, the cell is modified to produce GDP-fucose. More preferably, the cell is modified for enhanced GDP-fucose production. Said modification can be any one or more chosen from the group comprising knock-out of an UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase encoding gene, over-expression of a GDP-L-fucose synthase encoding gene, over-expression of a GDP-mannose 4,6-dehydratase encoding gene, over-expression of a mannose-1-phosphate guanylyltransferase encoding gene, over-expression of a phosphomannomutase encoding gene and over-expression of a mannose-6-phosphate isomerase encoding gene. Throughout the application, unless explicitly stated otherwise, the feature "enhanced" and/or "optimized" production preferably means that the modification(s) and/or metabolic engineering introduced in a cell as described herein results in a higher production yield compared to the wild type progenitor of said modified cell or metabolically engineered cell. For example, "an enhanced GDP-fucose production" preferably means that the intracellular production of GDP-fucose is higher in the modified cell compared to the wild type progenitor which does not contain these specific modifications.

Preferably, the cell in this context comprises a fucosylation pathway as described herein.

In another embodiment of the method and/or cell of the invention, at least one, preferably at least two, of said glycosyltransferases is a sialyltransferase and the cell is capable to synthesize CMP-Neu5Ac. The CMP-Neu5Ac can be provided by an enzyme expressed in the cell or by the metabolism of the cell. Such cell producing CMP-Neu5Ac can express an enzyme converting, e.g., sialic acid, which is to be added to the cell, to CMP-Neu5Ac. This enzyme may be a CMP-sialic acid synthetase, like the N-acetylneuraminase cytidyltransferase from several species including *Homo sapiens*, *Neisseria meningitidis*, and *Pasteurella multocida*. In a preferred embodiment of the method and/or cell of the present invention, said cell is capable of expressing at least one, preferably at least two, sialyltransferase(s) selected from alpha-2,3-sialyltransferases, alpha-2,6-sialyltransferases and alpha-2,8-sialyltransferases. Preferably, said sialyltransferases are selected from organisms like e.g. *Pasteurella* species like e.g. *Pasteurella multocida*, *Pasteurella dagmatis*, *Photobacterium* species like e.g. *Photobacterium damsela*, *Photobacterium* sp. JT-ISH-224, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, *Porphyromonas* species like e.g. *Porphyromonas catoniae*, *Streptococcus* species like e.g. *Streptococcus suis*, *Streptococcus agalactiae*, *Streptococcus entericus*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Haemophilus* species like e.g. *Haemophilus somnus*, *Haemophilus ducreyi*, *Haemophilus parahaemolyticus*, *Haemophilus parasuis*, *Vibrio* species, *Alistipes* species like e.g. *Alistipes* sp. CAG:268, *Alistipes* sp. AL-1, *Alistipes shahii*, *Alistipes*

timonensis, Actinobacillus species like e.g. Actinobacillus suis, Actinobacillus capsulatus, Homo sapiens, Mus musculus. In a more preferred embodiment of the method and/or cell of the present invention, said sialyltransferases are selected from the list comprising alpha-2,3-sialyltransferases and alpha-2,6-sialyltransferases.

5 Preferably, the cell is modified to produce CMP-Neu5Ac. More preferably, the cell is modified for enhanced CMP-Neu5Ac production. Said modification can be any one or more chosen from the group comprising knock-out of an N-acetylglucosamine-6-phosphate deacetylase, knock-out of a glucosamine-6-phosphate deaminase, over-expression of a sialate synthase encoding gene, and over-expression of an N-acetyl-D-glucosamine-2-epimerase encoding gene.

10 Preferably, the cell in this context comprises a sialylation pathway as described herein.

In another embodiment of the method and/or cell of the invention, at least one, preferably at least two, of said glycosyltransferases is an N-acetylglucosaminyltransferase and the cell is capable to synthesize UDP-GlcNAc. The UDP-GlcNAc can be provided by an enzyme expressed in the cell or by the metabolism of the cell. Such cell producing an UDP-GlcNAc can express enzymes converting, e.g. GlcNAc, which is to
 15 be added to the cell, to UDP-GlcNAc. These enzymes may be an N-acetyl-D-glucosamine kinase, an N-acetylglucosamine-6-phosphate deacetylase, a phosphoglucosamine mutase, and an N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase from several species including *Homo sapiens*, *Escherichia coli*. Alternatively, a cell can (preferably metabolically engineered to) express enzymes required for the synthesis of GlcNAc, such as glucosamine 6-phosphate
 20 N-acetyltransferase, phosphatase, glycosyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, and UDP-glucose 4-epimerase, preferably a glucosamine 6-phosphate N-acetyltransferase and a phosphatase (preferably a HAD-like phosphatase). In a preferred embodiment of the method and/or cell of the present invention, said cell is capable of expressing at least one, preferably at least two, N-acetylglucosaminyltransferase(s) selected from beta-1,3-N-
 25 acetylglucosaminyltransferases and beta-1,6-N-acetylglucosaminyltransferases. Preferably, said N-acetylglucosaminyltransferases are selected from organisms like e.g. Neisseria species, like e.g. Neisseria meningitidis, Neisseria lactamica, Neisseria polysaccharea, Neisseria elongata, Neisseria gonorrhoeae, Neisseria subflava, Pasteurella species like e.g. Pasteurella dagmatis, Neorhizobium species like e.g. Neorhizobium galegae, Haemophilus species like e.g. Haemophilus parainfluenzae, Haemophilus ducreyi,
 30 Homo sapiens, Mus musculus.

Preferably, the cell is modified to produce UDP-GlcNAc. More preferably, the cell is modified for enhanced UDP-GlcNAc production. Said modification can be any one or more chosen from the group comprising knock-out of an N-acetylglucosamine-6-phosphate deacetylase, over-expression of an L-glutamine—D-fructose-6-phosphate aminotransferase, over-expression of a phosphoglucosamine mutase, and over-
 35 expression of an N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase.

Preferably, the cell in this context comprises an N-acetylglucosamine carbohydrate pathway as described herein.

In another embodiment of the method and/or cell of the invention, at least one, preferably at least two, of said glycosyltransferases is a galactosyltransferase and the cell is capable to synthesize UDP-Gal. The UDP-Gal can be provided by an enzyme expressed in the cell or by the metabolism of the cell. Such cell producing UDP-Gal can express an enzyme converting, e.g. UDP-glucose, to UDP-Gal. This enzyme may be, e.g., the UDP-glucose-4-epimerase GalE like as known from several species including *Homo sapiens*, *Escherichia coli*, and *Rattus norvegicus*. In a preferred embodiment of the method and/or cell of the present invention, said cell is capable of expressing at least one, preferably at least two, galactosyltransferase(s) selected from beta-1,3-galactosyltransferases and beta-1,4-galactosyltransferases, and/or said cell is capable of expressing at least one, preferably at least two, galactosyltransferases selected from alpha-1,3-galactosyltransferases and alpha-1,4-galactosyltransferases. Preferably, said galactosyltransferases are chosen from organisms like e.g. *E. coli* species like e.g. *E. coli* O55:H7, *E. coli* DEC1B, *E. coli* DEC1D, *Neisseria* species like e.g. *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria elongata*, *Neisseria gonorrhoeae*, *Neisseria subflava*, *Kingella* species like e.g. *Kingella denitrificans*, *Brucella* species like e.g. *Brucella canis*, *Brucella suis*, *Salmonella* species, like e.g. *Salmonella enterica*, *Pseudogulbenkiana ferrooxidans*, *Corynebacterium glutamicum*, *Streptococcus* species, *Arabidopsis thaliana*, *Homo sapiens*, *Mus musculus*. Preferably, the cell is modified to produce UDP-Gal. More preferably, the cell is modified for enhanced UDP-Gal production. Said modification can be any one or more chosen from the group comprising knock-out of an bifunctional 5'-nucleotidase/UDP-sugar hydrolase encoding gene, knock-out of a galactose-1-phosphate uridylyltransferase encoding gene and over-expression of an UDP-glucose-4-epimerase encoding gene.

Preferably, the cell in this context comprises a galactosylation pathway as described herein.

In another embodiment of the method and/or cell of the invention, at least one, preferably at least two, of said glycosyltransferases is a N-acetylgalactosaminyltransferase and the cell is capable to synthesize UDP-GalNAc. The UDP-GalNAc can be provided by an enzyme expressed in the cell or by the metabolism of the cell. Such cell producing UDP-GalNAc can express an enzyme converting, e.g. UDP-glucose, to UDP-Gal. This enzyme may be, e.g., the UDP-glucose-4-epimerase GalE like as known from several species including *Homo sapiens*, *Escherichia coli*, and *Rattus norvegicus*. In a preferred embodiment of the method and/or cell of the present invention, said cell is capable of expressing at least one, preferably at least two, N-acetylgalactosaminyltransferase(s) selected from alpha-1,3- N-acetylgalactosaminyltransferases and beta-1,3- N-acetylgalactosaminyltransferases. Preferably, said N-acetylgalactosaminyltransferases are chosen from organisms like e.g. *Helicobacter* species like e.g. *Helicobacter mustelae*, *Haemophilus* species like e.g. *Haemophilus influenzae*, *Neisseria* species like e.g. *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria elongata*, *Neisseria*

gonorrhoeae, *Neisseria subflava*, *Rickettsia* species like e.g. *Rickettsia bellii*, *Rickettsia prowazekii*, *Rickettsia japonica*, *Rickettsia conorii*, *Rickettsia felis*, *Rickettsia massiliae*, *Homo sapiens*, *Mus musculus*. Preferably, the cell is modified to produce UDP-GalNAc. More preferably, the cell is modified for enhanced UDP-GalNAc production. Said modification can be any one or more chosen from the group comprising

5 knock-out of a bifunctional 5'-nucleotidase/UDP-sugar hydrolase encoding gene, knock-out of a galactose-1-phosphate uridylyltransferase encoding gene and over-expression of an UDP-glucose-4-epimerase encoding gene.

Preferably, the cell in this context comprises an N-acetylgalactosaminylation pathway as described herein.

10 In a preferred embodiment, the phosphatase as used herein is a HAD-like phosphatase. Phosphatases from the HAD superfamily and the HAD-like family are described in the art. Examples from these families can be found in the enzymes expressed from genes *yqaB*, *inhX*, *yniC*, *ybiV*, *yidA*, *ybjI*, *yigL* or *cof* from *Escherichia coli* or any one or more of e.g. the *E. coli* genes comprising *aphA*, *Cof*, *HisB*, *OtsB*, *SurE*, *Yaed*, *YcjU*, *YedP*, *YfbT*, *YidA*, *YigB*, *YihX*, *YniC*, *YqaB*, *YrbL*, *AppA*, *Gph*, *SerB*, *YbhA*, *YbiV*, *YbjL*, *Yfb*, *YieH*, *YjgI*, *YjjG*,

15 *YrfG* and *YbiU*; or *PsMupP* from *Pseudomonas putida*, *ScDOG1* from *S. cerevisiae* or *BsAraL* from *Bacillus subtilis* as described in WO18122225. One phosphatase that catalyses this reaction is identified in *Blastocladiella emersonii*. Phosphatases are generally not specific and the activity is generally not related to the family or structure. Other examples can thus be found in all phosphatase families. Specific phosphatases are easily identified and screened by well-known methods as described by Fahs et al. (ACS

20 Chem. Biol. 11(11), 2944-2961 (2016)).

Throughout the application, whenever a protein is disclosed, e.g. by referring to a SEQ ID NO, an unique database number (e.g. UNIPROT number) or by referring to the specific organism of origin, said protein embodiment can be preferably replaced with any, preferably all, of the following embodiments (and

25 hence said protein is considered to be disclosed according to all of the following embodiment):

- protein (e.g. by referring to a SEQ ID NO, an unique database number (e.g. UNIPROT number) or by referring to the specific organism of origin),
- a functional homologue, variant or derivative of said protein having at least 80% overall sequence identity to the full length of said protein,
- 30 - a functional fragment of said protein and having the same activity, or
- comprises a polypeptide comprising or consisting of an amino acid sequence having at least 80% sequence identity to the full-length amino acid sequence of said protein and having the same activity.

35 For example, when "*H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO: 05" is disclosed, said embodiment is preferably replaced with any, preferably all, of the following embodiments:

- H. pylori alpha-1,3-fucosyltransferase with SEQ ID NO: 05,
- alpha-1,3-fucosyltransferase comprising a polypeptide sequence according to SEQ ID NO: 05,
- a functional homologue, variant or derivative of SEQ ID NO: 05 having at least 80% overall sequence identity to the full length of SEQ ID NO: 05 and having alpha-1,3-fucosyltransferase activity,
- a functional fragment of SEQ ID NO: 05 and having alpha-1,3-fucosyltransferase activity, or
- comprises a polypeptide comprising or consisting of an amino acid sequence having at least 80% sequence identity to the full-length amino acid sequence of said SEQ ID NO: 05 and having alpha-1,3-fucosyltransferase activity.

In another embodiment of the method and/or cell of the invention, the cell is capable to synthesize any one of said nucleotide-sugars chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetyl fucosamine, UDP-N-acetyl fucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAc or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose. In a preferred embodiment, the cell is capable to synthesize two nucleotide-sugars. In a more preferred embodiment, the cell is capable to synthesize at least three nucleotide-activated sugars. In an even more preferred embodiment, the cell is capable to synthesize at least four nucleotide-activated sugars. In a most preferred embodiment, the cell is capable to synthesize at least five nucleotide-activated sugars. In another preferred embodiment, said cell is metabolically engineered for the production of a nucleotide-sugar. In another preferred embodiment, the cell is modified and/or engineered for the optimized production of a nucleotide-sugar, i.e. enhanced production of a nucleotide-sugar as described herein. In a more preferred embodiment, said cell is metabolically engineered for the production of two nucleotide-sugars. In an even more preferred embodiment, said cell is metabolically engineered for the production of three or more nucleotide-activated sugars.

In another embodiment of the method and/or cell of the present invention, the cell expresses one or more polypeptides chosen from the list comprising mannanose-6-phosphate isomerase, phosphomannomutase, mannanose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-

phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acetylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acetylneuraminate-9-phosphate synthase, N-acetylneuraminate-9-phosphate phosphatase, N-acetylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, UDP-N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and UDP-N-acetylgalactosamine pyrophosphorylase.

In a preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of lactose-based neutral non-fucosylated MMOs, is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a β -1,4-galactosyltransferase as described herein; and 2) capable to express an N-acetylglucosaminyltransferase as described herein, preferably a galactoside β -1,3-N-acetylglucosaminyltransferase; 3) optionally capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising an N-acetylglucosamine β -1,3-galactosyltransferase, an N-acetylglucosamine β -1,4-galactosyltransferase, an α -1,3-galactosyltransferase, an α -1,4-galactosyltransferase; and 4) optionally capable to express at least one, preferably at least two, N-acetylgalactosaminyltransferase(s) as described herein, chosen from the list comprising an α -1,3-N-acetylgalactosaminyltransferase and a β -1,3-N-acetylgalactosaminyltransferase and 5) capable to synthesize the nucleotide-sugar of each of said glycosyltransferases if present.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of lactose-based neutral fucosylated MMOs, is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a β -1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising α -1,2-fucosyltransferase, α -1,3-fucosyltransferase, α -1,4-fucosyltransferase and α -1,6-fucosyltransferase; 3) optionally capable to express an N-acetylglucosaminyltransferase as described herein, preferably a galactoside β -1,3-N-acetylglucosaminyltransferase; 4) optionally capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine β -1,3-galactosyltransferase, N-

acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase, alpha-1,4-galactosyltransferase; 5) optionally capable to express at least one, preferably at least two, N-acetylgalactosaminyltransferase(s) as described herein, chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase; 6) capable to
5 synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein, and 7) capable to synthesize the nucleotide-sugar of each of said glycosyltransferase if present.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of lactose-based sialylated non-fucosylated
10 MMOs, is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a b-1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, sialyltransferase(s) as described herein, chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase; 3) optionally capable to express an N-acetylglucosaminyltransferase as described herein,
15 preferably a galactoside beta-1,3-N-acetylglucosaminyltransferase; 4) optionally capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase, alpha-1,4-galactosyltransferase; 5) optionally capable to express at least one, preferably at least two, N-acetylgalactosaminyltransferase(s) as described
20 herein, chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase; 6) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein, and 7) capable to synthesize the nucleotide-sugar of each of said glycosyltransferase if present.

In another and/or additional preferred embodiment of the method and/or cell according to the invention,
25 the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of lactose-based sialylated fucosylated MMOs, is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a b-1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the
30 list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase; 3) capable to express at least one, preferably at least two, sialyltransferase(s) as described herein, chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase; 4) optionally capable to express a N-acetylglucosaminyltransferase as described herein, preferably a galactoside beta-1,3-N-acetylglucosaminyltransferase; 5) optionally capable to express at least one, preferably at least two,
35 galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine beta-

1,3-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase, alpha-1,4-galactosyltransferase; 6) optionally capable to express at least one, preferably at least two, N-acetylgalactosaminyltransferase(s) as described herein, chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylglactosaminyltransferase; 7) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein; 8) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein; and 9) capable to synthesize the nucleotide-sugar of each of said glycosyltransferase if present.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LNB-based neutral non-fucosylated MMOs, is able to produce LNB as described herein or is capable to take up LNB from the cultivation; and capable to synthesize UDP-Gal.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LNB-based neutral fucosylated MMOs, is 1) capable to take up LNB from the cultivation as described herein or is able to produce LNB as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase; 3) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein, and 4) optionally capable to produce UDP-galactose.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LNB-based sialylated non-fucosylated MMOs, is 1) capable to take up LNB from the cultivation as described herein or is able to produce LNB as described herein; and 2) capable to express at least one, preferably at least two sialyltransferase(s) as described herein, chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase; 3) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein, and 4) optionally capable to produce UDP-galactose.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LNB-based sialylated fucosylated MMOs, is 1) capable to take up LNB from the cultivation as described herein or is able to produce LNB as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-

1,4-fucosyltransferase and alpha-1,6-fucosyltransferase; 3) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein; 4) capable to express at least one, preferably at least two sialyltransferase(s) as described herein, chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase; 5) capable to synthesize
5 CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein; and 6) optionally capable to produce UDP-galactose.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LacNAc-based neutral non-fucosylated
10 MMOs, is able to produce LacNAc as described herein or is capable to take up LacNAc from the cultivation as described herein; and capable to synthesize UDP-Gal.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LacNAc-based neutral fucosylated MMOs, is
15 1) capable to take up LacNAc from the cultivation as described herein or is able to produce LacNAc as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase; 3) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein, and 4) optionally
20 capable to produce UDP-galactose.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different mammalian milk oligosaccharides according to the invention can be produced by providing a cell which, for the production of LacNAc-based sialylated non-fucosylated
25 MMOs, is 1) capable to take up LacNAc from the cultivation as described herein or is able to produce LacNAc as described herein; and 2) capable to express at least one, preferably at least two sialyltransferase(s) as described herein, chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase; 3) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein, and 4) optionally capable to produce UDP-galactose.

In another and/or additional preferred embodiment of the method and/or cell according to the invention, the mixture of at least three different oligosaccharides according to the invention can be produced by
30 providing a cell which, for the production of LacNAc-based sialylated fucosylated oligosaccharides, is 1) capable to take up LacNAc from the cultivation as described herein or is able to produce LacNAc as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase; 3) capable to
35 synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein; 4) capable to

express at least one, preferably at least two sialyltransferase(s) as described herein, chosen from the list comprising α -2,3-sialyltransferase, α -2,6-sialyltransferase and α -2,8-sialyltransferase; 5) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein; and 6) optionally capable to produce UDP-galactose.

- 5 In a more preferred embodiment of the method and/or cell according to the invention, a mixture of at least three different MMOs comprising neutral fucosylated lactose-based MMOs like e.g. fucosyllactose(s) and fucosylated LNT and/or LNnT structures like e.g. LNFP-I, LNFP-II, LNFP-III, LNFP-V, and no charged oligosaccharides can be produced by providing a cell which is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a b-
10 1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising α -1,2-fucosyltransferase, α -1,3-fucosyltransferase, α -1,4-fucosyltransferase and α -1,6-fucosyltransferase; 3) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein; 4) capable to express an N-acetylglucosaminyltransferase as described herein,
15 preferably a galactoside β -1,3-N-acetylglucosaminyltransferase; 5) capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine β -1,3-galactosyltransferase, N-acetylglucosamine β -1,4-galactosyltransferase, α -1,3-galactosyltransferase, α -1,4-galactosyltransferase; 6) capable to synthesize UDP-GlcNAc, preferably said cell has an N-acetylglucosaminylation pathway as defined herein; 7) capable to synthesize
20 UDP-Gal, preferably said cell has a galactosylation pathway as defined herein.

- In another more preferred embodiment of the method and/or cell according to the invention, a mixture of at least three different MMOs comprising charged lactose-based MMOs like e.g. sialyllactose(s) and sialylated lacto-N-triose and sialylated Lacto-N-tetraose(s) and/or sialylated lacto-N-neotetraose(s) and no fucosylated oligosaccharides can be produced by providing a cell which is 1) capable to take up lactose
25 from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a b-1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, sialyltransferase(s) as described herein, chosen from the list comprising α -2,3-sialyltransferase, α -2,6-sialyltransferase and α -2,8-sialyltransferase; 3) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein; 4) capable to express an
30 N-acetylglucosaminyltransferase as described herein, preferably a galactoside β -1,3-N-acetylglucosaminyltransferase; 5) capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine β -1,3-galactosyltransferase, N-acetylglucosamine β -1,4-galactosyltransferase, α -1,3-galactosyltransferase, α -1,4-galactosyltransferase; 6) capable to synthesize UDP-GlcNAc, preferably
35 said cell has an N-acetylglucosaminylation pathway as defined herein; 7) capable to synthesize UDP-Gal, preferably said cell has a galactosylation pathway as defined herein.

In another more preferred embodiment of the method and/or cell according to the invention, a mixture of at least three different MMOs comprising charged and neutral lactose-based MMOs like e.g. fucosyllactose(s), sialyllactose(s), LN3, fucosylated LNT and/or LNTnT, sialylated lacto-N-triose and sialylated Lacto-N-tetraose(s) and/or sialylated lacto-N-neotetraose(s) can be produced by providing a cell which is 1) capable to take up lactose from the cultivation as described herein or is able to produce lactose after uptake of glucose by the action of a β -1,4-galactosyltransferase as described herein; and 2) capable to express at least one, preferably at least two, sialyltransferase(s) as described herein, chosen from the list comprising α -2,3-sialyltransferase, α -2,6-sialyltransferase and α -2,8-sialyltransferase; 3) capable to synthesize CMP-sialic acid, preferably said cell has a sialylation pathway as defined herein; 4) capable to express at least one, preferably at least two, fucosyltransferase(s) as described herein, chosen from the list comprising α -1,2-fucosyltransferase, α -1,3-fucosyltransferase, α -1,4-fucosyltransferase and α -1,6-fucosyltransferase; 5) capable to synthesize GDP-fucose, preferably said cell has a fucosylation pathway as defined herein; 6) capable to express an N-acetylglucosaminyltransferase as described herein, preferably a galactoside β -1,3-N-acetylglucosaminyltransferase; 7) capable to express at least one, preferably at least two, galactosyltransferase(s) as described herein, chosen from the list comprising N-acetylglucosamine β -1,3-galactosyltransferase, N-acetylglucosamine β -1,4-galactosyltransferase, α -1,3-galactosyltransferase, α -1,4-galactosyltransferase; 8) capable to synthesize UDP-GlcNAc, preferably said cell has an N-acetylglucosaminylation pathway as defined herein; 9) capable to synthesize UDP-Gal, preferably said cell has a galactosylation pathway as defined herein.

Exemplary methods and cells according to the invention are described in the Examples section. It is emphasized that these examples show at least one way to produce the specific mixtures. The person skilled in the art will understand that any of the expressed enzymes can be replaced by another enzyme if it has the same catalytic activity, preferably to a similar extent, which can be readily assessed through routine experimentation wherein the activity of an enzyme is compared with the activity of a reference enzyme as disclosed herein (e.g. in vitro conversion of a substrate).

In a preferred embodiment of the method and/or cell of the invention, any one of said MMOs, more preferably all of said oligosaccharides, is/are translocated to the outside of the cell by a passive transport i.e. without means of an active transport system consuming energy from said cell.

In a preferred embodiment of the method and/or cell of the invention, the cell uses at least one precursor for the production of any one or more of said oligosaccharides. The term "precursor" should be understood as explained in the definitions as disclosed herein. In a more preferred embodiment, the cell uses two or more precursors for the production of any one or more of said oligosaccharides.

In a preferred embodiment of the method of the invention, the cultivation is fed with a precursor and/or acceptor for the synthesis of any one of said oligosaccharides in said mixture. The term "acceptor" should be understood as explained in the definitions as disclosed herein. In a further preferred embodiment of

the method, the cultivation is fed with at least two precursors and/or acceptors for the synthesis of any one or more, preferably all, of said oligosaccharides in the mixture. This can be useful if two or more glycosyltransferases of the same classification (e.g. 2-fucosyltransferases) are used which have a different affinity (e.g. one fucosyltransferase having affinity to lactose and the other fucosyltransferase having

5 affinity to LacNAc) for the production of a mixture of oligosaccharides according to the present invention.

In another embodiment of the method and/or cell as described herein, the cell is producing a precursor for the production of any one of said oligosaccharides. In a preferred embodiment, said cell is producing one or more precursors for the synthesis of said oligosaccharide mixture. In a more preferred embodiment, said cell is modified for optimized production of any one of said precursors for the synthesis

10 of any one of said oligosaccharides.

In a preferred embodiment of the method and/or cell of the invention, at least one precursor for the production of any one of said oligosaccharides is completely converted into any one of said oligosaccharides. In a more preferred embodiment, the cell completely converts any one of said precursors into any one of said oligosaccharides.

15 In another preferred embodiment of the method and/or cell of the invention, the cell is further metabolically engineered for

- i) modified expression of an endogenous membrane protein, and/or
- ii) modified activity of an endogenous membrane protein, and/or
- 20 iii) expression of a homologous membrane protein, and/or
- iv) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the secretion of any one of said MMOs outside said cell. Said cell can express one of said membrane proteins that is involved in the secretion of any one of said MMOs from said cell to the outside of said cell. Said cell can also express more than one of said membrane

25 proteins. Any one of said membrane proteins can translocate one or more of said MMOs to the outside of said cell. Said cell producing a mixture of at least three MMOs can translocate any one of said MMOs comprising passive diffusion, channel membrane proteins, membrane transporter proteins, membrane carrier proteins.

30 In another preferred embodiment of the method and/or cell of the invention, the cell is further metabolically engineered for

- i) modified expression of an endogenous membrane protein, and/or
- ii) modified activity of an endogenous membrane protein, and/or
- iii) expression of a homologous membrane protein, and/or
- 35 iv) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the uptake of a precursor and/or acceptor for the synthesis

of any one of said MMOs. In one embodiment, the cell expresses one of said membrane proteins that is involved in the uptake of any type of precursor and/or acceptor used in the synthesis of any one of said MMOs. In one embodiment, the cell expresses more than one of said membrane proteins, involved in the uptake of at least one of said precursors and/or acceptors. In one embodiment, the cell is modified for the uptake of more than one precursor and/or acceptor for the synthesis of any one of said MMOs. In a preferred embodiment, the cell is modified for the uptake of all the required precursors. In another preferred embodiment, the cell is modified for the uptake of all the acceptors.

In a more preferred embodiment of the method and/or cell of the invention, the membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β -barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators. In an even more preferred embodiment of the method and/or cell of the invention, the porters comprise MFS transporters, sugar efflux transporters and siderophore exporters. In another more preferred embodiment of the method and/or cell of the invention, the P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.

In another preferred embodiment of the method and/or cell of the invention, the membrane protein provides improved production of any one of said oligosaccharides, preferably all of said oligosaccharides. In an alternative and/or additional preferred embodiment of the method and/or cell of the invention, the membrane protein provides enabled efflux of any one of said oligosaccharides, preferably all of said oligosaccharides. In an alternative and/or additional preferred embodiment of the method and/or cell of the invention, the membrane protein provides enhanced efflux of any one of said oligosaccharides, preferably all of said oligosaccharides.

In a more preferred embodiment of the method and/or cell of the present invention, the cell expresses a membrane protein belonging to the family of MFS transporters like e.g. an MdfA polypeptide of the multidrug transporter MdfA family from species comprising *E. coli* (UniProt ID P0AEY8), *Cronobacter muytjensii* (UniProt ID A0A2T7ANQ9), *Citrobacter youngae* (UniProt ID D4BC23) and *Yokenella regensburgei* (UniProt ID G9Z5F4). In another more preferred embodiment of the method and/or cell of the present invention, the cell expresses a membrane protein belonging to the family of sugar efflux transporters like e.g. a SetA polypeptide of the SetA family from species comprising *E. coli* (UniProt ID P31675), *Citrobacter koseri* (UniProt ID A0A078LM16) and *Klebsiella pneumoniae* (UniProt ID A0A0C4MGS7). In another more preferred embodiment of the method and/or cell of the present invention, the cell expresses a membrane protein belonging to the family of siderophore exporters like e.g. the *E. coli* entS (UniProt ID P24077) and the *E. coli* iceT (UniProt ID A0A024L207). In another more preferred embodiment of the method and/or cell of the present invention, the cell expresses a membrane protein belonging to the family of ABC transporters like e.g. oppF from *E. coli* (UniProt ID P77737), ImrA from *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* (UniProt ID A0A1V0NEL4) and Blon_2475 from

Bifidobacterium longum subsp. *infantis* (UniProt ID B7GPD4).

In a preferred embodiment of the method and/or cell of the invention, the cell confers enhanced bacteriophage resistance. Said enhancement of bacteriophage resistance can be derived from reduced expression of an endogenous membrane protein and/or mutation of an endogenous membrane protein encoding gene. The term "phage insensitive" or "phage resistant" or "phage resistance" or "phage resistant profile" is understood to mean a bacterial strain that is less sensitive, and preferably insensitive to infection and/ or killing by phage and/ or growth inhibition. As used herein, the terms "anti-phage activity" or "resistant to infection by at least one phage" refers to an increase in resistance of a bacterial cell expressing a functional phage resistance system to infection by at least one phage family in comparison to a bacterial cell of the same species under the same developmental stage (e.g. culture state) which does not express a functional phage resistance system, as may be determined by e.g. bacterial viability, phage lysogeny, phage genomic replication and phage genomic degradation. The phage can be a lytic phage or a temperate (lysogenic) phage. Membrane proteins involved in bacteriophage resistance of a cell comprise OmpA, OmpC, OmpF, OmpT, BtuB, TolC, LamB, FhuA, TonB, FadL, Tsx, FepA, YncD, PhoE, and NfrA and homologs thereof.

In a preferred embodiment of the method and/or cell of the invention, the cell confers reduced viscosity. Reduced viscosity of a cell can be obtained by a modified cell wall biosynthesis. Cell wall biosynthesis can be modified comprising reduced or abolished synthesis of for example poly-N-acetyl-glucosamine, the enterobacterial common antigen, cellulose, colanic acid, core oligosaccharides, osmoregulated periplasmic glucans and glucosylglycerol, glycan, and trehalose.

According to another embodiment of the method and/or cell of the present invention, the cell is capable to produce phosphoenolpyruvate (PEP). In a preferred embodiment of the method and/or cell of the present invention, the cell is modified for enhanced production and/or supply of PEP compared to a non-modified progenitor.

In a preferred embodiment and as a means for enhanced production and/or supply of PEP, one or more PEP-dependent, sugar-transporting phosphotransferase system(s) is/are disrupted such as but not limited to: 1) the N-acetyl-D-glucosamine Npi-phosphotransferase (EC 2.7.1.193), which is for instance encoded by the nagE gene (or the cluster nagABCD) in *E. coli* or *Bacillus* species, 2) ManXYZ which encodes the Enzyme II Man complex (mannose PTS permease, protein-Npi- phosphohistidine-D-mannose phosphotransferase) that imports exogenous hexoses (mannose, glucose, glucosamine, fructose, 2-deoxyglucose, mannosamine, N-acetylglucosamine, etc.) and releases the phosphate esters into the cell cytoplasm, 3) the glucose-specific PTS transporter (for instance encoded by PtsG/Crr) which takes up glucose and forms glucose-6-phosphate in the cytoplasm, 4) the sucrose-specific PTS transporter which takes up sucrose and forms sucrose-6-phosphate in the cytoplasm, 5) the fructose-specific PTS transporter (for instance encoded by the genes fruA and fruB and the kinase fruK which takes up fructose and forms in a first step fructose-1-phosphate and in a second step fructose1,6 bisphosphate, 6) the lactose PTS

transporter (for instance encoded by *lacE* in *Lactococcus casei*) which takes up lactose and forms lactose-6-phosphate, 7) the galactitol-specific PTS enzyme which takes up galactitol and/or sorbitol and forms galactitol-1-phosphate or sorbitol-6-phosphate respectively, 8) the mannitol-specific PTS enzyme which takes up mannitol and/or sorbitol and forms mannitol-1-phosphate or sorbitol-6-phosphate respectively, and 9) the trehalose-specific PTS enzyme which takes up trehalose and forms trehalose-6-phosphate.

In another and/or additional preferred embodiment and as a means for enhanced production and/or supply of PEP, the full PTS system is disrupted by disrupting the *PtsIH/Crr* gene cluster. *PtsI* (Enzyme I) is a cytoplasmic protein that serves as the gateway for the phosphoenolpyruvate:sugar phosphotransferase system (PTS_{sugar}) of *E. coli* K-12. *PtsI* is one of two (*PtsI* and *PtsH*) sugar non-specific protein constituents of the PTS_{sugar} which along with a sugar-specific inner membrane permease effects a phosphotransfer cascade that results in the coupled phosphorylation and transport of a variety of carbohydrate substrates. *HPr* (histidine containing protein) is one of two sugar-non-specific protein constituents of the PTS_{sugar}. It accepts a phosphoryl group from phosphorylated Enzyme I (*PtsI*-P) and then transfers it to the *EIIA* domain of any one of the many sugar-specific enzymes (collectively known as Enzymes II) of the PTS_{sugar}. *Crr* or *EIIAGlc* is phosphorylated by PEP in a reaction requiring *PtsH* and *PtsI*.

In another and/or additional preferred embodiment, the cell is further modified to compensate for the deletion of a PTS system of a carbon source by the introduction and/or overexpression of the corresponding permease. These are e.g. permeases or ABC transporters that comprise but are not limited to transporters that specifically import lactose such as e.g. the transporter encoded by the *LacY* gene from *E. coli*, sucrose such as e.g. the transporter encoded by the *cscB* gene from *E. coli*, glucose such as e.g. the transporter encoded by the *galP* gene from *E. coli*, fructose such as e.g. the transporter encoded by the *frul* gene from *Streptococcus mutans*, or the Sorbitol/mannitol ABC transporter such as the transporter encoded by the cluster *SmoEFGK* of *Rhodobacter sphaeroides*, the trehalose/sucrose/maltose transporter such as the transporter encoded by the gene cluster *ThuEFGK* of *Sinorhizobium meliloti* and the N-acetylglucosamine/galactose/glucose transporter such as the transporter encoded by *NagP* of *Shewanella oneidensis*. Examples of combinations of PTS deletions with overexpression of alternative transporters are: 1) the deletion of the glucose PTS system, e.g. *ptsG* gene, combined with the introduction and/or overexpression of a glucose permease (e.g. *galP* of *glcP*), 2) the deletion of the fructose PTS system, e.g. one or more of the *fruB*, *fruA*, *fruK* genes, combined with the introduction and/or overexpression of fructose permease, e.g. *frul*, 3) the deletion of the lactose PTS system, combined with the introduction and/or overexpression of lactose permease, e.g. *LacY*, and/or 4) the deletion of the sucrose PTS system, combined with the introduction and/or overexpression of a sucrose permease, e.g. *cscB*.

In a further preferred embodiment, the cell is modified to compensate for the deletion of a PTS system of a carbon source by the introduction of carbohydrate kinases, such as glucokinase (EC 2.7.1.1, EC 2.7.1.2, EC 2.7.1.63), galactokinase (EC 2.7.1.6), and/or fructokinase (EC 2.7.1.3, EC 2.7.1.4). Examples of combinations of PTS deletions with overexpression of alternative transporters and a kinase are: 1) the

deletion of the glucose PTS system, e.g. ptsG gene, combined with the introduction and/or overexpression of a glucose permease (e.g. galP or glcP), combined with the introduction and/or overexpression of a glucokinase (e.g. glk), and/or 2) the deletion of the fructose PTS system, e.g. one or more of the fruB, fruA, fruK genes, combined with the introduction and/or overexpression of fructose permease, e.g. frul, combined with the introduction and/or overexpression of a fructokinase (e.g. frk or mak).

In another and/or additional preferred embodiment and as a means for enhanced production and/or supply of PEP, the cell is modified by the introduction of or modification in any one or more of the list comprising phosphoenolpyruvate synthase activity (EC: 2.7.9.2 encoded for instance in *E. coli* by ppsA), phosphoenolpyruvate carboxykinase activity (EC 4.1.1.32 or EC 4.1.1.49 encoded for instance in *Corynebacterium glutamicum* by PCK or in *E. coli* by pckA, resp.), phosphoenolpyruvate carboxylase activity (EC 4.1.1.31 encoded for instance in *E. coli* by ppc), oxaloacetate decarboxylase activity (EC 4.1.1.112 encoded for instance in *E. coli* by eda), pyruvate kinase activity (EC 2.7.1.40 encoded for instance in *E. coli* by pykA and pykF), pyruvate carboxylase activity (EC 6.4.1.1 encoded for instance in *B. subtilis* by pyc) and malate dehydrogenase activity (EC 1.1.1.38 or EC 1.1.1.40 encoded for instance in *E. coli* by maeA or maeB, resp.).

In a more preferred embodiment, the cell is modified to overexpress any one or more of the polypeptides comprising ppsA from *E. coli* (UniProt ID P23538), PCK from *C. glutamicum* (UniProt ID Q6F5A5), pckA from *E. coli* (UniProt ID P22259), eda from *E. coli* (UniProt ID P0A955), maeA from *E. coli* (UniProt ID P26616) and maeB from *E. coli* (UniProt ID P76558).

In another and/or additional preferred embodiment, the cell is modified to express any one or more polypeptide having phosphoenolpyruvate synthase activity, phosphoenolpyruvate carboxykinase activity, oxaloacetate decarboxylase activity, or malate dehydrogenase activity.

In another and/or additional preferred embodiment and as a means for enhanced production and/or supply of PEP, the cell is modified by a reduced activity of phosphoenolpyruvate carboxylase activity, and/or pyruvate kinase activity, preferably a deletion of the genes encoding for phosphoenolpyruvate carboxylase, the pyruvate carboxylase activity and/or pyruvate kinase.

In an exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate carboxykinase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate carboxylase gene, the overexpression of oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene, the overexpression of oxaloacetate decarboxylase combined with the deletion

of a phosphoenolpyruvate carboxylase gene, the overexpression of oxaloacetate decarboxylase combined with the deletion of a pyruvate carboxylase gene, the overexpression of malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene and/or the overexpression of malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene.

In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase, the overexpression of phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase, the overexpression of phosphoenolpyruvate carboxykinase combined with the overexpression of a malate dehydrogenase, the overexpression of oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase, the overexpression of phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase and/or the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase.

In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate kinase gene, the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of an oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of

phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene, the overexpression of phosphoenolpyruvate synthase combined the overexpression of oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene.

In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of an oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the

overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a phosphoenolpyruvate carboxylase gene.

- 5 In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of an oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate carboxylase gene.
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- 30 In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase combined with the deletion of a
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pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of an oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a phosphoenolpyruvate carboxylase gene.

In another exemplary embodiment, the cell is genetically modified by different adaptations such as the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of a malate dehydrogenase combined with the deletion

of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of an oxaloacetate decarboxylase combined with the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of a phosphoenolpyruvate carboxykinase and the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of a phosphoenolpyruvate carboxykinase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene, the overexpression of phosphoenolpyruvate synthase combined with the overexpression of an oxaloacetate decarboxylase and the overexpression of a malate dehydrogenase combined with the deletion of a pyruvate kinase gene and a pyruvate carboxylase gene and a phosphoenolpyruvate carboxylase gene.

According to another preferred embodiment of the method and/or cell of the invention, the cell comprises a modification for reduced production of acetate compared to a non-modified progenitor. Said modification can be any one or more chosen from the group comprising overexpression of an acetyl-coenzyme A synthetase, a full or partial knock-out or rendered less functional pyruvate dehydrogenase and a full or partial knock-out or rendered less functional lactate dehydrogenase.

In a further embodiment of the method and/or cell of the invention, the cell is modified in the expression or activity of at least one acetyl-coenzyme A synthetase like e.g. *acs* from *E. coli*, *S. cerevisiae*, *H. sapiens*, *M. musculus*. In a preferred embodiment, said acetyl-coenzyme A synthetase is an endogenous protein of the cell with a modified expression or activity, preferably said endogenous acetyl-coenzyme A synthetase is overexpressed; alternatively, said acetyl-coenzyme A synthetase is a heterologous protein that is heterogeneously introduced and expressed in said cell, preferably overexpressed. Said endogenous acetyl-coenzyme A synthetase can have a modified expression in the cell which also expresses a heterologous acetyl-coenzyme A synthetase. In a more preferred embodiment, the cell is modified in the expression or activity of the acetyl-coenzyme A synthetase *acs* from *E. coli* (UniProt ID P27550). In another and/or additional preferred embodiment, the cell is modified in the expression or activity of a functional homolog, variant or derivative of *acs* from *E. coli* (UniProt ID P27550) having at least 80 % overall sequence

identity to the full-length of said polypeptide from *E. coli* (UniProt ID P27550) and having acetyl-coenzyme A synthetase activity.

In an alternative and/or additional further embodiment of the method and/or cell of the invention, the cell is modified in the expression or activity of at least one pyruvate dehydrogenase like e.g. from *E. coli*,
5 *S. cerevisiae*, *H. sapiens* and *R. norvegicus*. In a preferred embodiment, the cell has been modified to have at least one partially or fully knocked out or mutated pyruvate dehydrogenase encoding gene by means generally known by the person skilled in the art resulting in at least one protein with less functional or being disabled for pyruvate dehydrogenase activity. In a more preferred embodiment, the cell has a full knock-out in the *poxB* encoding gene resulting in a cell lacking pyruvate dehydrogenase activity.

10 In an alternative and/or additional further embodiment of the method and/or cell of the invention, the cell is modified in the expression or activity of at least one lactate dehydrogenase like e.g. from *E. coli*, *S. cerevisiae*, *H. sapiens* and *R. norvegicus*. In a preferred embodiment, the cell has been modified to have at least one partially or fully knocked out or mutated lactate dehydrogenase encoding gene by means generally known by the person skilled in the art resulting in at least one protein with less functional or
15 being disabled for lactate dehydrogenase activity. In a more preferred embodiment, the cell has a full knock-out in the *ldhA* encoding gene resulting in a cell lacking lactate dehydrogenase activity.

According to another preferred embodiment of the method and/or cell of the invention, the cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-
20 acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIBAB-Man, EIIC-Man, EIID-Man, *ushA*, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase,
25 glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor *IclR*, *Ion* protease, glucose-specific translocating phosphotransferase enzyme IIBC component *ptsG*, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component *malX*, enzyme *IIAGlc*, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer
30 protein *FruA* and *FruB*, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacetyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

According to another preferred embodiment of the method and/or cell of the invention, the cell comprises a catabolic pathway for selected mono-, di- or oligosaccharides which is at least partially
35 inactivated, the mono-, di-, or oligosaccharides being involved in and/or required for the production of any one of said oligosaccharides from said mixture.

Another embodiment of the invention provides for a method and a cell wherein a mixture comprising at least three different MMOs is produced in and/or by a fungal, yeast, bacterial, insect, animal, plant and protozoan cell as described herein. The cell is chosen from the list comprising a bacterium, a yeast, a protozoan or a fungus, or, refers to a plant or animal cell. The latter bacterium preferably belongs to the phylum of the Proteobacteria or the phylum of the Firmicutes or the phylum of the Cyanobacteria or the phylum Deinococcus-Thermus. The latter bacterium belonging to the phylum Proteobacteria belongs preferably to the family Enterobacteriaceae, preferably to the species *Escherichia coli*. The latter bacterium preferably relates to any strain belonging to the species *Escherichia coli* such as but not limited to *Escherichia coli* B, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* K12, *Escherichia coli* Nissle.

More specifically, the latter term relates to cultivated *Escherichia coli* strains - designated as *E. coli* K12 strains - which are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine. Well-known examples of the *E. coli* K12 strains are K12 Wild type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention specifically relates to a mutated and/or transformed *Escherichia coli* cell or strain as indicated above wherein said *E. coli* strain is a K12 strain. More preferably, the *Escherichia coli* K12 strain is *E. coli* MG1655. The latter bacterium belonging to the phylum Firmicutes belongs preferably to the Bacilli, preferably Lactobacilliales, with members such as *Lactobacillus lactis*, *Leuconostoc mesenteroides*, or Bacillales with members such as from the genus *Bacillus*, such as *Bacillus subtilis* or, *B. amyloliquefaciens*. The latter Bacterium belonging to the phylum Actinobacteria, preferably belonging to the family of the Corynebacteriaceae, with members *Corynebacterium glutamicum* or *C. afermentans*, or belonging to the family of the Streptomycetaceae with members *Streptomyces griseus* or *S. fradiae*. The latter yeast preferably belongs to the phylum of the Ascomycota or the phylum of the Basidiomycota or the phylum of the Deuteromycota or the phylum of the Zygomycetes. The latter yeast belongs preferably to the genus *Saccharomyces* (with members like e.g. *Saccharomyces cerevisiae*, *S. bayanus*, *S. boulardii*), *Pichia* (with members like e.g. *Pichia pastoris*, *P. anomala*, *P. kluyveri*), *Komagataella*, *Hansenula*, *Kluyveromyces* (with members like e.g. *Kluyveromyces lactis*, *K. marxianus*, *K. thermotolerans*), *Debaromyces*, *Yarrowia* (like e.g. *Yarrowia lipolytica*) or *Starmerella* (like e.g. *Starmerella bombicola*). The latter yeast is preferably selected from *Pichia pastoris*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The latter fungus belongs preferably to the genus *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*. Plant cells include cells of flowering and non-flowering plants, as well as algal cells, for example *Chlamydomonas*, *Chlorella*, etc. Preferably, said plant is a tobacco, alfalfa, rice, tomato, cotton, rapeseed, soy, maize or corn plant. The latter animal cell is preferably derived from non-human mammals (e.g. cattle, buffalo, pig, sheep, mouse, rat), birds (e.g. chicken, duck, ostrich, turkey, pheasant), fish (e.g. swordfish, salmon, tuna, sea bass, trout, catfish), invertebrates (e.g. lobster, crab, shrimp, clams, oyster, mussel, sea urchin), reptiles (e.g. snake, alligator, turtle), amphibians (e.g. frogs) or insects (e.g. fly, nematode) or is a genetically modified cell line derived from human cells excluding

embryonic stem cells. Both human and non-human mammalian cells are preferably chosen from the list comprising an epithelial cell like e.g. a mammary epithelial cell, an embryonic kidney cell (e.g. HEK293 or HEK 293T cell), a fibroblast cell, a COS cell, a Chinese hamster ovary (CHO) cell, a murine myeloma cell like e.g. an N20, SP2/0 or YB2/0 cell, an NIH-3T3 cell, a non-mammary adult stem cell or derivatives thereof
 5 such as described in WO21067641. The latter insect cell is preferably derived from *Spodoptera frugiperda* like e.g. Sf9 or Sf21 cells, *Bombyx mori*, *Mamestra brassicae*, *Trichoplusia ni* like e.g. BTI-TN-5B1-4 cells or *Drosophila melanogaster* like e.g. *Drosophila* S2 cells. The latter protozoan cell preferably is a *Leishmania tarentolae* cell.

In a preferred embodiment of the method and/or cell of the invention, the cell is a viable Gram-negative
 10 bacterium that comprises a reduced or abolished synthesis of poly-N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides, Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or trehalose compared to a non-modified progenitor.

In a more preferred embodiment of the method and/or cell, said reduced or abolished synthesis of poly-
 15 N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides, Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or trehalose is provided by a mutation in any one or more glycosyltransferases involved in the synthesis of any one of said poly-N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides, Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or
 20 trehalose, wherein said mutation provides for a deletion or lower expression of any one of said glycosyltransferases. Said glycosyltransferases comprise glycosyltransferase genes encoding poly-N-acetyl-D-glucosamine synthase subunits, UDP-N-acetylglucosamine—undecaprenyl-phosphate N-acetylglucosaminophosphotransferase, Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose) transferase, UDP-N-acetyl-D-mannosaminuronic acid transferase, the glycosyltransferase genes encoding the
 25 cellulose synthase catalytic subunits, the cellulose biosynthesis protein, colanic acid biosynthesis glucuronosyltransferase, colanic acid biosynthesis galactosyltransferase, colanic acid biosynthesis fucosyltransferase, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, putative colanic biosynthesis glycosyl transferase, UDP-glucuronate:LPS(HepIII) glycosyltransferase, ADP-heptose—LPS heptosyltransferase 2, ADP-heptose:LPS heptosyltransferase 1, putative ADP-heptose:LPS
 30 heptosyltransferase 4, lipopolysaccharide core biosynthesis protein, UDP-glucose:(glucosyl)LPS α -1,2-glucosyltransferase, UDP-D-glucose:(glucosyl)LPS α -1,3-glucosyltransferase, UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6-D-galactosyltransferase, lipopolysaccharide glucosyltransferase I, lipopolysaccharide core heptosyltransferase 3, β -1,6-galactofuranosyltransferase, undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase, lipid IVA 4-amino-4-deoxy-L-
 35 arabinosyltransferase, bactoprenol glucosyl transferase, putative family 2 glycosyltransferase, the osmoregulated periplasmic glucans (OPG) biosynthesis protein G, OPG biosynthesis protein H,

glucosylglycerate phosphorylase, glycogen synthase, 1,4- α -glucan branching enzyme, 4- α -glucanotransferase and trehalose-6-phosphate synthase. In an exemplary embodiment, the cell is mutated in any one or more of the glycosyltransferases comprising pgaC, pgaD, rfe, rffT, rffM, bcsA, bcsB, bcsC, wcaA, wcaC, wcaE, wcaI, wcaJ, wcaL, waaH, waaF, waaC, waaU, waaZ, waaJ, waaO, waaB, waaS, waaG, waaQ, wbbI, arnC, arnT, yfdH, wbbK, opgG, opgH, ycjM, glgA, glgB, malQ, otsA and yaiP, wherein said mutation provides for a deletion or lower expression of any one of said glycosyltransferases.

In an alternative and/or additional preferred embodiment of the method and/or cell, said reduced or abolished synthesis of poly-N-acetyl-glucosamine (PNAG) is provided by over-expression of a carbon storage regulator encoding gene, deletion of a Na⁺/H⁺ antiporter regulator encoding gene and/or deletion of the sensor histidine kinase encoding gene.

The microorganism or cell as used herein is capable to grow on a monosaccharide, disaccharide, oligosaccharide, polysaccharide, polyol, glycerol, a complex medium including molasses, corn steep liquor, peptone, tryptone, yeast extract or a mixture thereof like e.g. a mixed feedstock, preferably a mixed monosaccharide feedstock like e.g. hydrolysed sucrose, as the main carbon source. With the term “complex medium” is meant a medium for which the exact constitution is not determined. With the term main is meant the most important carbon source for the bioproducts of interest, biomass formation, carbon dioxide and/or by-products formation (such as acids and/or alcohols, such as acetate, lactate, and/or ethanol), i.e. 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, 99 % of all the required carbon is derived from the above-indicated carbon source. In one embodiment of the invention, said carbon source is the sole carbon source for said organism, i.e. 100 % of all the required carbon is derived from the above-indicated carbon source. Common main carbon sources comprise but are not limited to glucose, glycerol, fructose, maltose, lactose, arabinose, malto-oligosaccharides, maltotriose, sorbitol, xylose, rhamnose, sucrose, galactose, mannose, methanol, ethanol, trehalose, starch, cellulose, hemi-cellulose, molasses, corn-steep liquor, high-fructose syrup, acetate, citrate, lactate and pyruvate. With the term complex medium is meant a medium for which the exact constitution is not determined. Examples are molasses, corn steep liquor, peptone, tryptone or yeast extract.

In a further preferred embodiment, the microorganism or cell described herein is using a split metabolism having a production pathway and a biomass pathway as described in WO2012/007481, which is herein incorporated by reference. Said organism can, for example, be genetically modified to accumulate fructose-6-phosphate by altering the genes selected from the phosphoglucisomerase gene, phosphofructokinase gene, fructose-6-phosphate aldolase gene, fructose isomerase gene, and/or fructose:PEP phosphotransferase gene.

According to another embodiment of the method of the invention, the conditions permissive to produce said oligosaccharides in the mixture comprise the use of a culture medium to cultivate a cell of present invention for the production of said oligosaccharide mixture wherein said culture medium lacks any precursor and/or acceptor for the production of any one of said oligosaccharides and is combined with a

further addition to said culture medium of at least one precursor and/or acceptor feed for the production of any one of said oligosaccharides, preferably for the production of all of said oligosaccharides in the mixture.

5 In a preferred embodiment, the method for the production of an oligosaccharide mixture as described herein comprises at least one of the following steps:

- i) Use of a culture medium comprising at least one precursor and/or acceptor;
- 10 ii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed;
- 15 iii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed and wherein preferably, the pH of said precursor and/or acceptor feed is set between 3 and 7 and wherein preferably, the temperature of said precursor and/or acceptor feed is kept between 20°C and 80°C;
- 20 iv) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- v) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein preferably, the pH of said feeding solution is set between 3 and 7 and wherein preferably, the temperature of said feeding solution is kept between 20°C and 80°C;
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30 said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

In another and/or additional preferred embodiment, the method for the production of an oligosaccharide mixture as described herein comprises at least one of the following steps:

- 35 i) Use of a culture medium comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic

meter);

ii) Adding to the culture medium at least one precursor and/or acceptor in one pulse or in a discontinuous (pulsed) manner wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed pulse(s);

iii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed in one pulse or in a discontinuous (pulsed) manner wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed pulse(s) and wherein preferably, the pH of said precursor and/or acceptor feed pulse(s) is set between 3 and 7 and wherein preferably, the temperature of said precursor and/or acceptor feed pulse(s) is kept between 20°C and 80°C;

iv) Adding at least one precursor and/or acceptor feed in a discontinuous (pulsed) manner to the culture medium over the course of 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 10 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

v) Adding at least one precursor and/or acceptor feed in a discontinuous (pulsed) manner to the culture medium over the course of 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 10 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein preferably, the pH of said feeding solution is set between 3 and 7 and wherein preferably, the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

In a further, more preferred embodiment, the method for the production of an oligosaccharide mixture as described herein comprises at least one of the following steps:

i) Use of a culture medium comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter);

ii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of

lactose per litre of initial reactor volume wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;

iii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed and wherein preferably the pH of said lactose feed is set between 3 and 7 and wherein preferably the temperature of said lactose feed is kept between 20°C and 80°C;

iv) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

v) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said feeding solution is set between 3 and 7 and wherein preferably the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

Preferably the lactose feed is accomplished by adding lactose from the beginning of the cultivation at a concentration of at least 5 mM, preferably in a concentration of 30, 40, 50, 60, 70, 80, 90, 100, 150 mM, more preferably at a concentration > 300 mM.

In another embodiment the lactose feed is accomplished by adding lactose to the culture medium in a concentration, such that throughout the production phase of the cultivation a lactose concentration of at least 5 mM, preferably 10 mM or 30 mM is obtained.

In a further embodiment of the methods described herein the host cells are cultivated for at least about 60, 80, 100, or about 120 hours or in a continuous manner.

In a preferred embodiment, a carbon source is provided, preferably sucrose, in the culture medium for 3 or more days, preferably up to 7 days; and/or provided, in the culture medium, at least 100, advantageously at least 105, more advantageously at least 110, even more advantageously at least 120 grams of sucrose per litre of initial culture volume in a continuous manner, so that the final volume of the culture medium is not more than three-fold, advantageously not more than two-fold, more advantageously less than two-fold of the volume of the culturing medium before the culturing.

Preferably, when performing the method as described herein, a first phase of exponential cell growth is provided by adding a carbon source, preferably glucose or sucrose, to the culture medium before the precursor, preferably lactose, is added to the cultivation in a second phase.

In another preferred embodiment of the method of present invention, a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein only a carbon-based substrate, preferably glucose or sucrose, is added to the cultivation.

In another preferred embodiment of the method of present invention, a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein a carbon-based substrate, preferably glucose or sucrose, and a precursor, preferably lactose, are added to the cultivation.

In an alternative preferable embodiment, in the method as described herein, the precursor is added already in the first phase of exponential growth together with the carbon-based substrate.

In another preferred embodiment of the method, the culture medium contains at least one precursor selected from the group comprising lactose, galactose, fucose, sialic acid, GlcNAc, GalNAc, lacto-N-biose (LNB) and N-acetyllactosamine (LacNAc).

According to the present invention, the method as described herein preferably comprises a step of separating of any one or more of said oligosaccharides, preferably all of said oligosaccharides, from said cultivation.

The terms "separating from said cultivation" means harvesting, collecting, or retrieving any one of said oligosaccharides, preferably all of said oligosaccharides, from the cell and/or the medium of its growth.

Any one of said oligosaccharides can be separated in a conventional manner from the aqueous culture medium, in which the cell was grown. In case said oligosaccharide is still present in the cells producing the oligosaccharide mixture, conventional manners to free or to extract said oligosaccharide out of the cells can be used, such as cell destruction using high pH, heat shock, sonication, French press, homogenization, enzymatic hydrolysis, chemical hydrolysis, solvent hydrolysis, detergent, hydrolysis,... The culture medium and/or cell extract together and separately can then be further used for separating said oligosaccharide. This preferably involves clarifying said oligosaccharide containing mixture to remove suspended

particulates and contaminants, particularly cells, cell components, insoluble metabolites and debris produced by culturing the genetically modified cell. In this step, said oligosaccharide containing mixture can be clarified in a conventional manner. Preferably, said oligosaccharide containing mixture is clarified by centrifugation, flocculation, decantation and/or filtration. Another step of separating said oligosaccharide from said oligosaccharide containing mixture preferably involves removing substantially all the proteins, as well as peptides, amino acids, RNA and DNA and any endotoxins and glycolipids that could interfere with the subsequent separation step, from said oligosaccharide containing mixture, preferably after it has been clarified. In this step, proteins and related impurities can be removed from said oligosaccharide containing mixture in a conventional manner. Preferably, proteins, salts, by-products, colour, endotoxins and other related impurities are removed from said oligosaccharide containing mixture by ultrafiltration, nanofiltration, two-phase partitioning, reverse osmosis, microfiltration, activated charcoal or carbon treatment, treatment with non-ionic surfactants, enzymatic digestion, tangential flow high-performance filtration, tangential flow ultrafiltration, electrophoresis (e.g. using slab-polyacrylamide or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE)), affinity chromatography (using affinity ligands including e.g. DEAE-Sepharose, poly-L-lysine and polymyxin-B, endotoxin-selective adsorber matrices), ion exchange chromatography (such as but not limited to cation exchange, anion exchange, mixed bed ion exchange, inside-out ligand attachment), hydrophobic interaction chromatography and/or gel filtration (i.e., size exclusion chromatography), particularly by chromatography, more particularly by ion exchange chromatography or hydrophobic interaction chromatography or ligand exchange chromatography. With the exception of size exclusion chromatography, proteins and related impurities are retained by a chromatography medium or a selected membrane, said oligosaccharide remains in the said oligosaccharide containing mixture.

In a further preferred embodiment, the methods as described herein also provide for a further purification of any one or more of said mammalian milk oligosaccharide(s) from the MMO mixture. A further purification of said MMO(s) may be accomplished, for example, by use of (activated) charcoal or carbon, nanofiltration, ultrafiltration, electrophoresis, enzymatic treatment or ion exchange to remove any remaining DNA, protein, LPS, endotoxins, or other impurity. Alcohols, such as ethanol, and aqueous alcohol mixtures can also be used. Another purification step is accomplished by crystallization, evaporation or precipitation of the product. Another purification step is to dry, e.g. spray dry, lyophilize, spray freeze dry, freeze spray dry, band dry, belt dry, vacuum band dry, vacuum belt dry, drum dry, roller dry, vacuum drum dry or vacuum roller dry the produced oligosaccharide(s).

In an exemplary embodiment, the separation and purification of at least one, preferably all, of the produced MMOs is made in a process, comprising the following steps in any order:

- a) contacting the cultivation or a clarified version thereof with a nanofiltration membrane with a molecular weight cut-off (MWCO) of 600-3500 Da ensuring the retention of produced MMO(s)

and allowing at least a part of the proteins, salts, by-products, colour and other related impurities to pass,

- b) conducting a diafiltration process on the retentate from step a), using said membrane, with an aqueous solution of an inorganic electrolyte, followed by optional diafiltration with pure water to remove excess of the electrolyte,
- c) and collecting the retentate enriched in the MMO(s) in the form of a salt from the cation of said electrolyte.

In an alternative exemplary embodiment, the separation and purification of at least one, preferably all, of the produced MMOs is made in a process, comprising the following steps in any order: subjecting the cultivation or a clarified version thereof to two membrane filtration steps using different membranes, wherein

- one membrane has a molecular weight cut-off of between about 300 to about 500 Dalton, and
- the other membrane as a molecular weight cut-off of between about 600 to about 800 Dalton.

In an alternative exemplary embodiment, the separation and purification of at least one, preferably all, of the produced MMOs is made in a process, comprising the following steps in any order comprising the step of treating the cultivation or a clarified version thereof with a strong cation exchange resin in H⁺-form and a weak anion exchange resin in free base form.

In an alternative exemplary embodiment, the separation and purification of at least one, preferably all, of the produced oligosaccharides is made in the following way. The cultivation comprising the produced oligosaccharides, biomass, medium components and contaminants is applied to the following purification steps:

- i) separation of biomass from the cultivation,
- ii) cationic ion exchanger treatment for the removal of positively charged material,
- iii) anionic ion exchanger treatment for the removal of negatively charged material,
- iv) nanofiltration step and/or electrodialysis step,

wherein a purified solution comprising the produced MMO(s) at a purity of greater than or equal to 80 percent is provided. Optionally the purified solution is dried by any one or more drying steps chosen from the list comprising spray drying, lyophilization, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying and vacuum roller drying.

In an alternative exemplary embodiment, the separation and purification of at least one, preferably all, of the produced MMOs is made in a process, comprising the following steps in any order: enzymatic

treatment of the cultivation; removal of the biomass from the cultivation; ultrafiltration; nanofiltration; and a column chromatography step. Preferably such column chromatography is a single column or a multiple column. Further preferably the column chromatography step is simulated moving bed chromatography. Such simulated moving bed chromatography preferably comprises i) at least 4 columns, wherein at least one column comprises a weak or strong cation exchange resin; and/or ii) four zones I, II, III and IV with different flow rates; and/or iii) an eluent comprising water; and/or iv) an operating temperature of 15 degrees to 60 degrees centigrade.

In a specific embodiment, the present invention provides the produced MMO or MMO mixture which is dried to powder by any one or more drying steps chosen from the list comprising spray drying, lyophilization, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying and vacuum roller drying, wherein the dried powder contains < 15 percent -wt. of water, preferably < 10 percent -wt. of water, more preferably < 7 percent -wt. of water, most preferably < 5 percent -wt. of water.

In a third aspect, the present invention provides for the use of a metabolically engineered cell as described herein for the production of a mixture comprising at least three different mammalian milk oligosaccharides.

For identification of the oligosaccharides in the mixture comprising at least three different MMOs produced in the cell as described herein, the monomeric building blocks (e.g. the monosaccharide or glycan unit composition), the anomeric configuration of side chains, the presence and location of substituent groups, degree of polymerization/molecular weight and the linkage pattern can be identified by standard methods known in the art, such as, e.g. methylation analysis, reductive cleavage, hydrolysis, GC-MS (gas chromatography-mass spectrometry), MALDI-MS (Matrix-assisted laser desorption/ionization-mass spectrometry), ESI-MS (Electrospray ionization-mass spectrometry), HPLC (High-Performance Liquid chromatography with ultraviolet or refractive index detection), HPAEC-PAD (High-Performance Anion-Exchange chromatography with Pulsed Amperometric Detection), CE (capillary electrophoresis), IR (infrared)/Raman spectroscopy, and NMR (Nuclear magnetic resonance) spectroscopy techniques. The crystal structure can be solved using, e.g., solid-state NMR, FT-IR (Fourier transform infrared spectroscopy), and WAXS (wide-angle X-ray scattering). The degree of polymerization (DP), the DP distribution, and polydispersity can be determined by, e.g., viscosimetry and SEC (SEC-HPLC, high performance size-exclusion chromatography). To identify the monomeric components of the saccharide methods such as, e.g. acid-catalyzed hydrolysis, HPLC (high performance liquid chromatography) or GLC (gas-liquid chromatography) (after conversion to alditol acetates) may be used. To determine the glycosidic linkages, the saccharide is methylated with methyl iodide and strong base in DMSO, hydrolysis is performed, a reduction to partially methylated alditols is achieved, an acetylation to methylated alditol acetates is performed, and the analysis is carried out by GLC/MS (gas-liquid chromatography coupled with mass spectrometry). To determine the oligosaccharide sequence, a partial

depolymerization is carried out using an acid or enzymes to determine the structures. To identify the anomeric configuration, the oligosaccharide is subjected to enzymatic analysis, e.g. it is contacted with an enzyme that is specific for a particular type of linkage, e.g., beta-galactosidase, or alpha-glucosidase, etc., and NMR may be used to analyze the products.

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Products comprising the oligosaccharide mixture

In some embodiments, an oligosaccharide mixture produced as described herein is incorporated into a food (e.g, human food or feed), dietary supplement, pharmaceutical ingredient, cosmetic ingredient or medicine. In some embodiments, the oligosaccharide mixture is mixed with one or more ingredients
10 suitable for food, feed, dietary supplement, pharmaceutical ingredient, cosmetic ingredient or medicine. In some embodiments, the dietary supplement comprises at least one prebiotic ingredient and/or at least one probiotic ingredient.

A "prebiotic" is a substance that promotes growth of microorganisms beneficial to the host, particularly microorganisms in the gastrointestinal tract. In some embodiments, a dietary supplement provides
15 multiple prebiotics, including the oligosaccharide mixture produced and/or purified by a process disclosed in this specification, to promote growth of one or more beneficial microorganisms. Examples of prebiotic ingredients for dietary supplements include other prebiotic molecules (such as HMOs) and plant polysaccharides (such as inulin, pectin, b- glucan and xylooligosaccharide). A "probiotic" product typically contains live microorganisms that replace or add to gastrointestinal microflora, to the benefit of the
20 recipient. Examples of such microorganisms include Lactobacillus species (for example, L. acidophilus and L. bulgaricus), Bifidobacterium species (for example, B. animalis, B. longum and B. infantis (e.g., Bi-26)), and Saccharomyces boulardii. In some embodiments, an oligosaccharide mixture produced and/or purified by a process of this specification is orally administered in combination with such microorganism. Examples of further ingredients for dietary supplements include disaccharides (such as lactose),
25 monosaccharides (such as glucose and galactose), thickeners (such as gum arabic), acidity regulators (such as trisodium citrate), water, skimmed milk, and flavourings.

In some embodiments, the oligosaccharide mixture is incorporated into a human baby food (e.g., infant formula). Infant formula is generally a manufactured food for feeding to infants as a complete or partial substitute for human breast milk. In some embodiments, infant formula is sold as a powder and prepared
30 for bottle- or cup-feeding to an infant by mixing with water. The composition of infant formula is typically designed to be roughly mimic human breast milk. In some embodiments, an oligosaccharide mixture produced and/or purified by a process in this specification is included in infant formula to provide nutritional benefits similar to those provided by the oligosaccharides in human breast milk. In some embodiments, the oligosaccharide mixture is mixed with one or more ingredients of the infant formula.
35 Examples of infant formula ingredients include non-fat milk, carbohydrate sources (e.g., lactose), protein sources (e.g., whey protein concentrate and casein), fat sources (e.g., vegetable oils - such as palm, high

oleic safflower oil, rapeseed, coconut and/or sunflower oil; and fish oils), vitamins (such as vitamins A, Bb, Bi2, C and D), minerals (such as potassium citrate, calcium citrate, magnesium chloride, sodium chloride, sodium citrate and calcium phosphate) and possibly HMOs. Such HMOs may include, for example, DiFL, lacto-N-triose II, LNT, LNnT, lacto-N-fucopentaose I, lacto-N-neofucopentaose, lacto-N-fucopentaose II, 5 lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-neofucopentaose V, lacto-N-difucohexaose I, lacto-N-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-N-hexaose and lacto-N-neohexaose.

In some embodiments, the one or more infant formula ingredients comprise non-fat milk, a carbohydrate source, a protein source, a fat source, and/or a vitamin and mineral.

10 In some embodiments, the one or more infant formula ingredients comprise lactose, whey protein concentrate and/or high oleic safflower oil.

In some embodiments, the oligosaccharide mixture's concentration in the infant formula is approximately the same concentration as the oligosaccharide's concentration generally present in human breast milk. In some embodiments, the concentration of each of the single oligosaccharides in the mixture of 15 oligosaccharides in the infant formula is approximately the same concentration as the concentration of that oligosaccharide generally present in human breast milk.

In some embodiments, the oligosaccharide mixture is incorporated into a feed preparation, wherein said feed is chosen from the list comprising petfood, animal milk replacer, veterinary product, post weaning feed, or creep feed.

20 Each embodiment disclosed in the context of one aspect of the invention, is also disclosed in the context of all other aspects of the invention, unless explicitly stated otherwise.

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic 25 chemistry and nucleic acid chemistry and hybridization described above and below are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, purification steps are performed according to the manufacturer's specifications.

Further advantages follow from the specific embodiments, the examples and the attached drawings. It goes without saying that the abovementioned features and the features which are still to be explained 30 below can be used not only in the respectively specified combinations, but also in other combinations or on their own, without departing from the scope of the present invention.

The present invention relates to following specific embodiments:

1. A metabolically engineered cell producing a mixture of at least three different mammalian milk 35 oligosaccharides, wherein said cell
 - expresses at least two glycosyltransferases, and

- is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferase.
2. Cell according to embodiment 1, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.
 3. Cell according to any one of embodiments 1 and 2, wherein said cell produces a mixture of charged and neutral mammalian milk oligosaccharides.
 4. Cell according to any one of embodiments 1 to 3, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.
 5. Cell according to any one of embodiments 1 to 4, wherein said cell produces four or more different mammalian milk oligosaccharides.
 6. Cell according to any one of embodiments 1 to 5, wherein any one of said glycosyltransferase is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases.
 7. Cell according to any one of embodiments 1 to 6 wherein said cell is modified in the expression or activity of at least one of said glycosyltransferases.
 8. Cell according to any one of embodiments 1 to 7 wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).
 9. Cell according to any one of embodiments 1 to 8 wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).
 10. Cell according to any one of embodiments 1 to 9 wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).
 11. Cell according to any one of embodiments 1 to 10 wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).
 12. Cell according to any one of embodiments 1 to 11, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylglactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.
 13. Cell according to any one of embodiments 1 to 12, wherein at least one of said mammalian milk oligosaccharides is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated,

contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

14. Cell according to any one of embodiments 1 to 13, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.

15. Cell according to any one of embodiments 1 to 14, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.

16. Cell according to any one of embodiments 1 to 15, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.

17. Cell according to any one of embodiments 1 to 16, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.

18. Cell according to any one of embodiments 1 to 17, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

ii) modified activity of an endogenous membrane protein, and/or

iii) expression of a homologous membrane protein, and/or

iv) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the secretion of any one of said mammalian milk oligosaccharides from said mixture outside said cell.

19. Cell according to any one of embodiments 1 to 18, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

ii) modified activity of an endogenous membrane protein, and/or

iii) expression of a homologous membrane protein, and/or

iv) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the uptake of a precursor for the synthesis of any one of said mammalian milk oligosaccharides.

20. Cell according to any one of embodiments 1 to 19, wherein said cell is producing a precursor for the synthesis of any one of said mammalian milk oligosaccharides.

21. Cell according to any one of embodiments 1 to 20, wherein any one of said mammalian milk oligosaccharides is a human milk oligosaccharide, preferably all said oligosaccharides are human milk oligosaccharides.

22. A method to produce a mixture of at least three different mammalian milk oligosaccharides by a cell, the method comprising the steps of:

a. providing a cell expressing at least two glycosyltransferases and capable to synthesize one or

more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases, and

b. cultivating said cell under conditions permissive to express said glycosyltransferases and to synthesize said nucleotide-sugar(s),

5 c. preferably, separating at least one of said mammalian milk oligosaccharides from said cultivation.

23. Method according to embodiment 22, wherein said cell is a metabolically engineered cell according to any one of embodiments 1 to 21.

24. Method according to any one of embodiments 22 and 23, wherein said cell produces a mixture of charged and neutral mammalian milk oligosaccharides.

10 25. Method according to any one of embodiments 22 to 24, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.

26. Method according to any one of embodiments 22 to 25, wherein said cell produces four or more different mammalian milk oligosaccharides.

15 27. Method according to any one of embodiments 22 to 26, wherein any one of said glycosyltransferases is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases.

20 28. Method according to any one of embodiments 22 to 27 wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).

29. Method according to any one of embodiments 22 to 28 wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).

25 30. Method according to any one of embodiments 22 to 29 wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).

31. Method according to any one of embodiments 22 to 30 wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).

30 32. Method according to any one of embodiments 22 to 31, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylglactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.

35 33. Method according to any one of embodiments 22 to 32, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.

34. Method according to any one of embodiments 22 to 33, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.
35. Method according to any one of embodiments 22 to 34, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.
36. Method according to any one of embodiments 22 to 35, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.
37. Method according to any one of embodiments 22 to 36, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.
38. Method according to any one of embodiments 22 to 37, wherein said cell uses at least one precursor for the synthesis of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the synthesis of any one or more of said oligosaccharides.
39. Method according to any one of embodiments 22 to 38, wherein said cell is producing a precursor for the synthesis of any one of said mammalian milk oligosaccharides.
40. The method according to any one of embodiments 22 to 39, wherein said precursor for the synthesis of any one of said mammalian milk oligosaccharides is completely converted into any one of said mammalian milk oligosaccharides.
41. Method according to any one of embodiments 22 to 40, wherein any one of said mammalian milk oligosaccharides as a human milk oligosaccharide, preferably all of said mammalian milk oligosaccharides are human milk oligosaccharides.
42. The method according to any one of embodiments 22 to 41, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, reverse osmosis, microfiltration, activated charcoal or carbon treatment, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.
43. The method according to any one of embodiments 22 to 42 further comprising purification of any one of said mammalian milk oligosaccharides from said cell.
44. The method according to any one of embodiments 22 to 43, wherein said purification comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, spray drying or lyophilization.
45. The cell according to any one of embodiments 1 to 21 or method according to any one of

embodiments 22 to 44, wherein said cell is selected from the group consisting of microorganism, plant, or animal cells, preferably said microorganism is a bacterium, fungus or a yeast, preferably said plant is a rice, cotton, rapeseed, soy, maize or corn plant, preferably said animal is an insect, fish, bird or non-human mammal, preferably said animal cell is a mammalian cell line.

- 5 46. The cell according to any one of embodiments 1 to 21 and 45, or method according to any one of embodiments 22 to 45, wherein said cell is a cell of a bacterium, preferably of an *Escherichia coli* strain, more preferably of an *Escherichia coli* strain which is a K-12 strain, even more preferably the *Escherichia coli* K-12 strain is *E. coli* MG1655.
- 10 47. The cell according to any one of embodiments 1 to 21 and 45, or method according to any one of embodiments 22 to 45, wherein said cell is a yeast cell.
48. Use of a cell according to any one of embodiment 1 to 21, 45 to 47, or method according to any one of embodiment 22 to 47 for the production of a mixture of at least three different mammalian milk oligosaccharides.
- 15 Moreover, the present invention relates to the following preferred specific embodiments:
1. A metabolically engineered cell producing a mixture of at least three different mammalian milk oligosaccharides, wherein said cell
 - is metabolically engineered for the production of said mixture, and
 - expresses at least two glycosyltransferases, and
 - 20 - is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferase.
 2. Cell according to preferred embodiment 1, wherein said cell is capable to express, preferably expresses at least five glycosyltransferases, preferably at least six, more preferably at least seven glycosyltransferases.
 - 25 3. Cell according to any one of preferred embodiment 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.
 4. Cell according to any one of preferred embodiment 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged oligosaccharides.
 - 30 5. Cell according to any one of preferred embodiment 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.
 6. Cell according to any one of preferred embodiment 1 or 2, wherein said mixture comprises, consists or consists essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.
 - 35

7. Cell according to any one of preferred embodiment 1 or 2, wherein said mixture comprises, consists or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.
8. Cell according to any one of preferred embodiments 1 to 7, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.
9. Cell according to any one of specific embodiment 1 to 8, wherein said cell comprises multiple copies of the same coding DNA sequence encoding for one protein.
10. Cell according to any one of preferred embodiments 1 to 9, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.
11. Cell according to any one of preferred embodiments 1 to 10, wherein said cell produces four or more different mammalian milk oligosaccharides.
12. Cell according to any one of preferred embodiments 1 to 11, wherein any one of said glycosyltransferase is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases,
 - preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,
 - preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,
 - preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,
 - preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,
 - preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,
 - preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,

- preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase.
13. Cell according to any one of preferred embodiments 1 to 12, wherein said cell is modified in the expression or activity of at least one of said glycosyltransferases.
 - 5 14. Cell according to any one of preferred embodiments 1 to 13, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).
 15. Cell according to any one of preferred embodiments 1 to 14, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).
 - 10 16. Cell according to any one of preferred embodiments 1 to 15, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).
 17. Cell according to any one of preferred embodiments 1 to 16, wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).
 - 15 18. Cell according to any one of preferred embodiment 1 to 17, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine (UDP-GalNAc).
 19. Cell according to any one of preferred embodiment 1 to 18, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine
 - 20 (UDP-ManNAc).
 20. Cell according to any one of preferred embodiments 1 to 19, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, 25 CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose, UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylfucosamine, UDP-N-acetylfucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), 30 UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose.
 21. Cell according to any one of preferred embodiments 1 to 20, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, 35 phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-

- phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, UDP-N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.
22. Cell according to any one of preferred embodiments 1 to 21, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.
23. Cell according to any one of preferred embodiments 1 to 22, wherein at least one of said mammalian milk oligosaccharides is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.
24. Cell according to any one of preferred embodiments 1 to 23, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.
25. Cell according to any one of preferred embodiments 1 to 24, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.
26. Cell according to any one of preferred embodiments 1 to 25, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.
27. Cell according to any one of preferred embodiments 1 to 26, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.
28. Cell according to any one of preferred embodiments 1 to 27, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the production of any one or more of said oligosaccharides, said precursor(s) being fed to the cell from the cultivation medium.
29. Cell according to any one of preferred embodiments 1 to 28, wherein said cell is producing at least one precursor for the production of any one of said oligosaccharides.

30. Cell according to any one of preferred embodiments 1 to 29, wherein said at least one precursor for the production of any one of said oligosaccharides is completely converted into any one of said oligosaccharides.
31. Cell according to any one of preferred embodiments 1 to 30, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.
32. Cell according to any one of preferred embodiments 1 to 31, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or
 - iv) expression of a heterologous membrane protein,
- wherein said membrane protein is involved in the secretion of any one of said mammalian milk oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.
33. Cell according to any one of preferred embodiments 1 to 32, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or
 - iv) expression of a heterologous membrane protein,
- wherein said membrane protein is involved in the uptake of a precursor and/or acceptor for the synthesis of any one of said mammalian milk oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.
34. Cell according to any one of preferred embodiment 32 or 33, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β -barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators,
- preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters,
- preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.
35. Cell according to any one of preferred embodiment 32 to 34, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said

oligosaccharides.

36. Cell according to any one of preferred embodiment 1 to 35, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).

37. Cell according to any one of preferred embodiment 1 to 36, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.

38. Cell according to preferred embodiment 37, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIBAB-Man, EIIC-Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, Icn protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacetyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

39. Cell according to any one of preferred embodiment 1 to 38, wherein the cell is capable to produce phosphoenolpyruvate (PEP).

40. Cell according to any one of preferred embodiment 1 to 39, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.

41. Cell according to any one of preferred embodiments 1 to 40, wherein any one of said mammalian milk oligosaccharides is a human milk oligosaccharide, preferably all said oligosaccharides are human milk oligosaccharides.

42. A method to produce a mixture of at least three different mammalian milk oligosaccharides by a cell, the method comprising the steps of:

a. providing a cell, preferably a single cell, expressing at least two glycosyltransferases and capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases, and

b. cultivating said cell under conditions permissive to express said glycosyltransferases and to synthesize said nucleotide-sugar(s), resulting in said cell producing at least three different

mammalian milk oligosaccharides,

- c. preferably, separating at least one of said mammalian milk oligosaccharides from said cultivation, more preferably, separating all of said mammalian milk oligosaccharides from said cultivation.

43. Method according to preferred embodiment 42, wherein said cell is capable to express, preferably expresses at least five glycosyltransferases, preferably at least six, more preferably at least seven glycosyltransferases.

44. Method according to any one of preferred embodiment 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.

45. Method according to any one of preferred embodiment 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged oligosaccharides.

46. Method according to any one of preferred embodiment 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.

47. Method according to any one of preferred embodiment 42 or 43, wherein said mixture comprises, consists or consists essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.

48. Method according to any one of preferred embodiment 42 or 43, wherein said mixture comprises, consists or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.

49. Method according to any one of preferred embodiment 42 to 48, wherein said cell is a metabolically engineered cell according to any one of embodiments 1 to 41.

50. Method according to preferred embodiment 49, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.

51. Method according to any one of preferred embodiment 49 or 50, wherein said cell comprises multiple copies of the same coding DNA sequence encoding for one protein.

52. Method according to any one of preferred embodiments 42 to 51, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.

53. Method according to any one of preferred embodiments 42 to 52, wherein said cell produces four or more different mammalian milk oligosaccharides.

54. Method according to any one of preferred embodiments 42 to 53, wherein any one of said glycosyltransferases is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-

acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases,

- preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,

- preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,

- preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,

- preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,

- preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,

- preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,

- preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase,

- preferably, said cell is modified in the expression or activity of at least one of said glycosyltransferases.

55. Method according to any one of preferred embodiments 42 to 54, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).

56. Method according to any one of preferred embodiments 42 to 55, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).

57. Method according to any one of preferred embodiments 42 to 56, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).

58. Method according to any one of preferred embodiments 42 to 57, wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-

galactose (UDP-Gal).

59. Method according to any one of preferred embodiments 42 to 58, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine (UDP-GalNAc).

5 60. Method according to any one of preferred embodiments 42 to 59, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine (UDP-ManNAc).

61. Method according to any one of preferred embodiments 42 to 60, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, 10 UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylfucosamine, UDP-N-acetylfucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP- 15 L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.

20 62. Method according to any one of preferred embodiments 42 to 61, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, 25 glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, UDP-N-acetylglucosamine 4- 35 epimerase, N-acetylgalactosamine kinase and UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.

63. Method according to any one of preferred embodiments 42 to 62, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.
64. Method according to any one of preferred embodiments 42 to 63, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.
65. Method according to any one of preferred embodiments 42 to 64, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.
66. Method according to any one of preferred embodiments 42 to 65, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.
67. Method according to any one of preferred embodiments 42 to 66, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.
68. Method according to any one of preferred embodiments 42 to 67, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.
69. Method according to any one of preferred embodiments 42 to 68, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the production of any one or more of said oligosaccharides, said precursor(s) being fed to the cell from the cultivation medium.
70. Method according to any one of preferred embodiments 42 to 69, wherein said cell is producing at least one precursor for the production of any one of said mammalian milk oligosaccharides.
71. Method according to any one of preferred embodiments 42 to 70, wherein said at least one precursor for the production of any one of said mammalian milk oligosaccharides is completely converted into any one of said mammalian milk oligosaccharides.
72. Method according to any one of preferred embodiments 42 to 71, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.
73. Method according to any one of preferred embodiments 42 to 72, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or

iv) expression of a heterologous membrane protein,
wherein said membrane protein is involved in the secretion of any one of said mammalian milk oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.

74. Method according to any one of preferred embodiments 42 to 73, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

ii) modified activity of an endogenous membrane protein, and/or

iii) expression of a homologous membrane protein, and/or

iv) expression of a heterologous membrane protein,
wherein said membrane protein is involved in the uptake of a precursor and/or acceptor for the synthesis of any one of said mammalian milk oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.

75. Method according to any one of preferred embodiment 73 or 74, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β -barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators,

preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters,

preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.

76. Method according to any one of preferred embodiment 73 to 75, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said oligosaccharides.

77. Method according to any one of preferred embodiment 42 to 76, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).

78. Method according to any one of preferred embodiment 42 to 77, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.

79. Method according to preferred embodiment 78, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine

kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIAB-Man, EIIC-Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, Ion protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

80. Method according to any one of preferred embodiment 42 to 79, wherein the cell is capable to produce phosphoenolpyruvate (PEP).

81. Method according to any one of preferred embodiment 42 to 80, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.

82. Method according to any one of preferred embodiments 42 to 81, wherein any one of said mammalian milk oligosaccharides as a human milk oligosaccharide, preferably all of said mammalian milk oligosaccharides are human milk oligosaccharides.

83. Method according to any one of preferred embodiment 42 to 82, wherein said conditions comprise:

- use of a culture medium comprising at least one precursor and/or acceptor for the production of any one of said oligosaccharides, and/or
- adding to the culture medium at least one precursor and/or acceptor feed for the production of any one of said oligosaccharides.

84. Method according to any one of preferred embodiment 42 to 83, the method comprising at least one of the following steps:

- i) Use of a culture medium comprising at least one precursor and/or acceptor;
- ii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed;
- iii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed and

wherein preferably, the pH of said precursor and/or acceptor feed is set between 3 and 7 and wherein preferably, the temperature of said precursor and/or acceptor feed is kept between 20°C and 80°C;

- iv) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- v) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein preferably, the pH of said feeding solution is set between 3 and 7 and wherein preferably, the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

85. Method according to any one of preferred embodiment 42 to 83, the method comprising at least one of the following steps:

- i) Use of a culture medium comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter);
- ii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said lactose feed;
- iii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said lactose feed and wherein preferably the pH of said lactose feed is set between 3 and 7 and wherein preferably the temperature of said lactose feed is kept between 20°C and 80°C;
- iv) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

- v) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L and wherein preferably the pH of said feeding solution is set between 3 and 7 and wherein preferably the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

86. Method according to preferred embodiment 85, wherein the lactose feed is accomplished by adding lactose from the beginning of the cultivation in a concentration of at least 5 mM, preferably in a concentration of 30, 40, 50, 60, 70, 80, 90, 100, 150 mM, more preferably in a concentration > 300 mM.

87. Method according to any one of preferred embodiment 85 or 86, wherein said lactose feed is accomplished by adding lactose to the cultivation in a concentration, such, that throughout the production phase of the cultivation a lactose concentration of at least 5 mM, preferably 10 mM or 30 mM is obtained.

88. Method according to any one of preferred embodiment 42 to 87, wherein the host cells are cultivated for at least about 60, 80, 100, or about 120 hours or in a continuous manner.

89. Method according to any one of preferred embodiment 42 to 88, wherein said cell is cultivated in a culture medium comprising a carbon source comprising a monosaccharide, disaccharide, oligosaccharide, polysaccharide, polyol, glycerol, a complex medium including molasses, corn steep liquor, peptone, tryptone or yeast extract; preferably, wherein said carbon source is chosen from the list comprising glucose, glycerol, fructose, sucrose, maltose, lactose, arabinose, malto-oligosaccharides, maltotriose, sorbitol, xylose, rhamnose, galactose, mannose, methanol, ethanol, trehalose, starch, cellulose, hemi-cellulose, molasses, corn-steep liquor, high-fructose syrup, acetate, citrate, lactate and pyruvate.

90. Method according to any one of preferred embodiment 42 to 89, wherein the culture medium contains at least one precursor selected from the group comprising lactose, galactose, fucose, sialic acid, GlcNAc, GalNAc, lacto-N-biose (LNB), N-acetyllactosamine (LacNAc).

91. Method according to any one of preferred embodiment 42 to 90, wherein a first phase of exponential

cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium before the precursor, preferably lactose, is added to the culture medium in a second phase.

92. Method according to any one of preferred embodiment 42 to 91, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein only a carbon-based substrate, preferably glucose or sucrose, is added to the culture medium.

93. Method according to any one of preferred embodiment 42 to 92, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein a carbon-based substrate, preferably glucose or sucrose, and a precursor, preferably lactose, are added to the culture medium.

94. Method according to any one of preferred embodiments 42 to 93, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, two-phase partitioning, reverse osmosis, microfiltration, activated charcoal or carbon treatment, treatment with non-ionic surfactants, enzymatic digestion, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.

95. Method according to any one of preferred embodiments 42 to 94 further comprising purification of any one of said mammalian milk oligosaccharides from said cell.

96. Method according to any one of preferred embodiments 95, wherein said purification comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration, electrophoresis, enzymatic treatment or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, lyophilization, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying or vacuum roller drying.

97. Cell according to any one of preferred embodiments 1 to 41 or method according to any one of preferred embodiments 42 to 96, wherein said cell is a bacterium, fungus, yeast, a plant cell, an animal cell, or a protozoan cell,

- preferably said bacterium is an *Escherichia coli* strain, more preferably an *Escherichia coli* strain which is a K-12 strain, even more preferably the *Escherichia coli* K-12 strain is *E. coli* MG1655,
- preferably said fungus belongs to a genus chosen from the group comprising *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*,
- preferably said yeast belongs to a genus chosen from the group comprising *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Komagataella*, *Hansenula*, *Yarrowia*, *Starmerella*, *Kluyveromyces* or *Debaromyces*,

- preferably said plant cell is an algal cell or is derived from tobacco, alfalfa, rice, tomato, cotton, rapeseed, soy, maize, or corn plant,
- preferably said animal cell is derived from non-human mammals, birds, fish, invertebrates, reptiles, amphibians or insects or is a genetically modified cell line derived from human cells excluding embryonic stem cells, more preferably said human and non-human mammalian cell is an epithelial cell, an embryonic kidney cell, a fibroblast cell, a COS cell, a Chinese hamster ovary (CHO) cell, a murine myeloma cell, an NIH-3T3 cell, a non-mammary adult stem cell or derivatives thereof, more preferably said insect cell is derived from *Spodoptera frugiperda*, *Bombyx mori*, *Mamestra brassicae*, *Trichoplusia ni* or *Drosophila melanogaster*,
- preferably said protozoan cell is a *Leishmania tarentolae* cell.

98. Cell according to preferred embodiment 97, or method according to preferred embodiment 97, wherein said cell is a viable Gram-negative bacterium that comprises a reduced or abolished synthesis of poly-N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides, Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or trehalose compared to a non-modified progenitor.

99. Use of a cell according to any one of preferred embodiment 1 to 41, 97, 98, or method according to any one of preferred embodiment 42 to 98 for the production of a mixture of at least three different mammalian milk oligosaccharides.

The invention will be described in more detail in the examples and the attached figures, in which **Figure 1** shows the chromatogram plot obtained of a whole broth sample taken from the LSTc producing *E. coli* strain S2 (Table 8) expressing the α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 with SEQ ID NO 26 and analyzed via the Dionex method for presence of oligosaccharides, as described in Example 1. Peaks indicated represent following glycans (with retention times): 1, lactose (5.325 min); 2, LN3 (6.825 min); 3, LNNt (9.625 min); 4, sialic acid (20.359 min); 5, LSTc (30.809 min); 6, 6'SL (31.984 min). **Figure 2** shows the chromatogram plot obtained of a whole broth sample taken from an LSTd producing *E. coli* strain S5 (Table 9) expressing the α -2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and analyzed via the Dionex method for presence of oligosaccharides, as described in Example 1. Peaks indicated represent following glycans (with retention times): 1, lactose (5.334 min); 2, LN3 (6.825 min); 3, LNNt (9.567 min); 4, sialic acid (20.017 min); 5, LSTd (31.709 min); 6, 3'SL (33.050 min).

The following examples will serve as further illustration and clarification of the present invention and are not intended to be limiting.

ExamplesExample 1. Materials and Methods *Escherichia coli*Media

The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). The minimal medium used in the cultivation experiments in 96-well plates or in shake flasks contained 2.00 g/L NH₄Cl, 5.00 g/L (NH₄)₂SO₄, 2.993 g/L KH₂PO₄, 7.315 g/L K₂HPO₄, 8.372 g/L MOPS, 0.5 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 30 g/L sucrose or 30 g/L glycerol, 1 ml/L vitamin solution, 100 µl/L molybdate solution, and 1 mL/L selenium solution. As specified in the respective examples, 0.30 g/L sialic acid, 20 g/L lactose, 20 g/L LacNAc and/or 20 g/L LNB were additionally added to the medium as precursor(s). The minimal medium was set to a pH of 7 with 1M KOH. Vitamin solution consisted of 3.6 g/L FeCl₂·4H₂O, 5 g/L CaCl₂·2H₂O, 1.3 g/L MnCl₂·2H₂O, 0.38 g/L CuCl₂·2H₂O, 0.5 g/L CoCl₂·6H₂O, 0.94 g/L ZnCl₂, 0.0311 g/L H₃BO₄, 0.4 g/L Na₂EDTA·2H₂O and 1.01 g/L thiamine·HCl. The molybdate solution contained 0.967 g/L NaMoO₄·2H₂O. The selenium solution contained 42 g/L SeO₂.

The minimal medium for fermentations contained 6.75 g/L NH₄Cl, 1.25 g/L (NH₄)₂SO₄, 2.93 g/L KH₂PO₄ and 7.31 g/L K₂HPO₄, 0.5 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 30 g/L sucrose or 30 g/L glycerol, 1 mL/L vitamin solution, 100 µL/L molybdate solution, and 1 mL/L selenium solution with the same composition as described above. As specified in the respective examples, 0.30 g/L sialic acid, 20 g/L lactose, 20 g/L LacNAc and/or 20 g/L LNB were additionally added to the medium as precursor(s).

Complex medium was sterilized by autoclaving (121°C, 21 min) and minimal medium by filtration (0.22 µm Sartorius). When necessary, the medium was made selective by adding an antibiotic: e.g. chloramphenicol (20 mg/L), carbenicillin (100 mg/L), spectinomycin (40 mg/L) and/or kanamycin (50 mg/L).

Plasmids

pKD46 (Red helper plasmid, Ampicillin resistance), pKD3 (contains an FRT-flanked chloramphenicol resistance (cat) gene), pKD4 (contains an FRT-flanked kanamycin resistance (kan) gene), and pCP20 (expresses FLP recombinase activity) plasmids were obtained from Prof. R. Cunin (Vrije Universiteit Brussel, Belgium in 2007). Plasmids were maintained in the host *E. coli* DH5alpha (F⁻, phi80d/*lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, lambda⁻, *thi-1*, *gyrA96*, *relA1*) bought from Invitrogen.

Strains and mutations

Escherichia coli K12 MG1655 [λ⁻, F⁻, rph-1] was obtained from the Coli Genetic Stock Center (US), CGSC Strain#: 7740, in March 2007. Gene disruptions, gene introductions and gene replacements were performed using the technique published by Datsenko and Wanner (PNAS 97 (2000), 6640-6645). This

technique is based on antibiotic selection after homologous recombination performed by lambda Red recombinase. Subsequent catalysis of a flippase recombinase ensures removal of the antibiotic selection cassette in the final production strain. Transformants carrying a Red helper plasmid pKD46 were grown in 10 mL LB media with ampicillin, (100 mg/L) and L-arabinose (10 mM) at 30 °C to an OD_{600nm} of 0.6. The cells were made electrocompetent by washing them with 50 mL of ice-cold water, a first time, and with 1mL ice cold water, a second time. Then, the cells were resuspended in 50 µL of ice-cold water. Electroporation was done with 50 µL of cells and 10-100 ng of linear double-stranded-DNA product by using a Gene Pulser™ (BioRad) (600 Ω, 25 µFD, and 250 volts). After electroporation, cells were added to 1 mL LB media incubated 1 h at 37 °C, and finally spread onto LB-agar containing 25 mg/L of chloramphenicol or 50 mg/L of kanamycin to select antibiotic resistant transformants. The selected mutants were verified by PCR with primers upstream and downstream of the modified region and were grown in LB-agar at 42°C for the loss of the helper plasmid. The mutants were tested for ampicillin sensitivity. The linear ds-DNA amplicons were obtained by PCR using pKD3, pKD4 and their derivatives as template. The primers used had a part of the sequence complementary to the template and another part complementary to the side on the chromosomal DNA where the recombination must take place. For the genomic knock-out, the region of homology was designed 50-nt upstream and 50-nt downstream of the start and stop codon of the gene of interest. For the genomic knock-in, the transcriptional starting point (+1) had to be respected. PCR products were PCR-purified, digested with DpnI, re-purified from an agarose gel, and suspended in elution buffer (5 mM Tris, pH 8.0). Selected mutants were transformed with pCP20 plasmid, which is an ampicillin and chloramphenicol resistant plasmid that shows temperature- sensitive replication and thermal induction of FLP synthesis. The ampicillin-resistant transformants were selected at 30°C, after which a few were colony purified in LB at 42 °C and then tested for loss of all antibiotic resistance and of the FLP helper plasmid. The gene knock outs and knock ins are checked with control primers.

In an example for GDP-fucose production, the mutant strain was derived from *E. coli* K12 MG1655 comprising knock-outs of the *E. coli wcaJ* and *thyA* genes and genomic knock-ins of constitutive transcriptional units containing a sucrose transporter like e.g. CscB from *E. coli* W with SEQ ID NO 01, a fructose kinase like e.g. Frk originating from *Zymomonas mobilis* with SEQ ID NO 02 and a sucrose phosphorylase like e.g. BaSPSP originating from *Bifidobacterium adolescentis* with SEQ ID NO 03. For production of fucosylated oligosaccharides, the mutant GDP-fucose production strain was additionally modified with expression plasmids comprising constitutive transcriptional units for an alpha-1,2-fucosyltransferase like e.g. HpFutC from *H. pylori* with SEQ ID NO 04 and/or an alpha-1,3-fucosyltransferase like e.g. HpFucT from *H. pylori* with SEQ ID NO 05 and with a constitutive transcriptional unit for a selective marker like e.g. the *E. coli thyA* with SEQ ID NO 07. The constitutive transcriptional units of the fucosyltransferase genes could also be present in the mutant *E. coli* strain via genomic knock-ins. GDP-fucose production can further be optimized in the mutant *E. coli* strain by genomic knock-outs

of the *E. coli* genes comprising *glgC*, *agp*, *pfkA*, *pfkB*, *pgi*, *arcA*, *iclR*, *pgi* and *lon* as described in WO2016075243 and WO2012007481. GDP-fucose production can additionally be optimized comprising genomic knock-ins of constitutive transcriptional units for a mannose-6-phosphate isomerase like e.g. *manA* from *E. coli* with SEQ ID NO 08, a phosphomannomutase like e.g. *manB* from *E. coli* with SEQ ID NO 09, a mannose-1-phosphate guanylyltransferase like e.g. *manC* from *E. coli* with SEQ ID NO 10, a GDP-mannose 4,6-dehydratase like e.g. *gmd* from *E. coli* with SEQ ID NO 11 and a GDP-L-fucose synthase like e.g. *fcl* from *E. coli* with SEQ ID NO 12. GDP-fucose production can also be obtained by genomic knock-outs of the *E. coli* *fucK* and *fucI* genes and genomic knock-ins of constitutive transcriptional units containing a fucose permease like e.g. *fucP* from *E. coli* with SEQ ID NO 13 and a bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase like e.g. *fkp* from *Bacteroides fragilis* with SEQ NO ID 14. If the mutant strains producing GDP-fucose were intended to make fucosylated lactose structures, the strains were additionally modified with genomic knock-outs of the *E. coli* *LacZ*, *LacY* and *LacA* genes and with a genomic knock-in of a constitutive transcriptional unit for a lactose permease like e.g. the *E. coli* *LacY* with SEQ ID NO 15.

Alternatively, and/or additionally, production of GDP-fucose and/or fucosylated structures can further be optimized in the mutant *E. coli* strains with genomic knock-ins of a constitutive transcriptional unit comprising a membrane transporter protein like e.g. *MdfA* from *Cronobacter muytjensii* (UniProt ID A0A2T7ANQ9), *MdfA* from *Citrobacter youngae* (UniProt ID D4BC23), *MdfA* from *E. coli* (UniProt ID POAEY8), *MdfA* from *Yokenella regensburgei* (UniProt ID G9Z5F4), *iceT* from *E. coli* (UniProt ID A0A024L207) or *iceT* from *Citrobacter youngae* (UniProt ID D4B8A6).

In an example for sialic acid production, the mutant strain was derived from *E. coli* K12 MG1655 comprising knock-outs of the *E. coli* *nagA* and *nagB* genes and genomic knock-ins of constitutive transcriptional units containing a glucosamine 6-phosphate N-acetyltransferase like e.g. *GNA1* from *Saccharomyces cerevisiae* with SEQ ID NO 16, an N-acetylglucosamine 2-epimerase like e.g. *AGE* from *Bacteroides ovatus* with SEQ ID NO 17 and an N-acetylneuraminate (Neu5Ac) synthase like e.g. *NeuB* from *Neisseria meningitidis* with SEQ ID NO 18. Sialic acid production can further be optimized in the mutant *E. coli* strain with genomic knock-outs of any one or more of the *E. coli* genes comprising *nagC*, *nagD*, *nagE*, *nanA*, *nanE*, *nanK*, *manX*, *manY* and *manZ* as described in WO18122225 and/or genomic knock-outs of the *E. coli* genes comprising any one or more of *nanT*, *poxB*, *ldhA*, *adhE*, *aldB*, *pflA*, *pflC*, *ybiY*, *ackA* and/or *pta* and with genomic knock-ins of constitutive transcriptional units comprising an L-glutamine—D-fructose-6-phosphate aminotransferase like e.g. the mutant *glmS**54 from *E. coli* with SEQ ID NO 19 differing from the wild-type *E. coli* *glmS* by an A39T, an R250C and an G472S mutation and a phosphatase like any one or more of e.g. *yqaB* from *E. coli* with SEQ ID NO 20 or the *E. coli* genes comprising *aphA*, *Cof*, *HisB*, *OtsB*, *SurE*, *Yaed*, *YcjU*, *YedP*, *YfbT*, *YidA*, *YigB*, *YihX*, *YniC*, *YrbL*, *AppA*, *Gph*, *SerB*, *YbhA*, *YbiV*, *YbjL*, *Yfb*, *YieH*, *YjgL*, *YjjG*, *YrfG* and *YbiU* or *PsMupP* from *Pseudomonas putida*, *ScDOG1* from *S. cerevisiae* and *BsAraL* from *Bacillus subtilis* as described in WO18122225 and an acetyl-CoA synthetase like e.g. *acs* from

E. coli (UniProt ID P27550). Sialic acid production can also be obtained by knock-outs of the *E. coli* *nagA* and *nagB* genes and genomic knock-ins of constitutive transcriptional units containing a phosphoglucosamine mutase like e.g. glmM from *E. coli* with SEQ ID NO 42, an N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase like e.g. glmU from *E. coli* with SEQ ID NO 43, an UDP-N-acetylglucosamine 2-epimerase like e.g. NeuC from *Campylobacter jejuni* with SEQ ID NO 21 and an N-acetylneuraminate synthase like e.g. NeuB from *N. meningitidis* with SEQ ID NO 18. Also in this mutant strain, sialic acid production can further be optimized with genomic knock-ins of constitutive transcriptional units comprising an L-glutamine—D-fructose-6-phosphate aminotransferase like e.g. the mutant glmS*54 from *E. coli* with SEQ ID NO 19 and a phosphatase like e.g. yqaB from *E. coli* with SEQ ID NO 20 or the *E. coli* genes comprising aphA, Cof, HisB, OtsB, SurE, Yaed, YcjU, YedP, YfbT, YidA, YigB, YihX, YniC, YrbL, AppA, Gph, SerB, YbhA, YbiV, YbjL, Yfb, YieH, YjgL, YjjG, YrfG and YbiU or PsMupP from *Pseudomonas putida*, ScDOG1 from *S. cerevisiae* and BsAraL from *Bacillus subtilis* as described in WO18122225 and an acetyl-CoA synthetase like e.g. acs from *E. coli* (UniProt ID P27550).

Alternatively, and/or additionally, sialic acid production can be obtained by genomic knock-ins of constitutive transcriptional units containing a bifunctional UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase like e.g. from *Mus musculus* (strain C57BL/6J) (UniProt ID Q91WG8), an N-acetylneuraminate-9-phosphate synthetase like e.g. from *Pseudomonas* sp. UW4 (UniProt ID K9NPH9) and an N-acetylneuraminate-9-phosphatase like e.g. from *Candidatus Magnetomorum* sp. HK-1 (UniProt ID KPA15328.1) or from *Bacteroides thetaiotaomicron* (UniProt ID Q8A712).

Alternatively, and/or additionally, sialic acid production can be obtained by genomic knock-ins of constitutive transcriptional units containing a phosphoglucosamine mutase like e.g. glmM from *E. coli* (UniProt ID P31120), an N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase like e.g. glmU from *E. coli* (UniProt ID P0ACC7), a bifunctional UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase like e.g. from *M. musculus* (strain C57BL/6J) (UniProt ID Q91WG8), an N-acetylneuraminate-9-phosphate synthetase like e.g. from *Pseudomonas* sp. UW4 (UniProt ID K9NPH9) and an N-acetylneuraminate-9-phosphatase like e.g. from *Candidatus Magnetomorum* sp. HK-1 (UniProt ID KPA15328.1) or from *Bacteroides thetaiotaomicron* (UniProt ID Q8A712).

For sialylated oligosaccharide production, the sialic acid production strains further need to express an N-acetylneuraminate cytidyltransferase like e.g. NeuA from *Pasteurella multocida* with SEQ ID NO 22, and a beta-galactoside alpha-2,3-sialyltransferase like e.g. PmultST3 from *P. multocida* (UniProt ID Q9CLP3) or a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity like SEQ ID NO 23, NmeniST3 from *N. meningitidis* (SEQ ID NO 24) or PmultST2 from *P. multocida* subsp. *multocida* str. Pm70 (GenBank NO. AAK02592.1), a beta-galactoside alpha-2,6-sialyltransferase like e.g. PdST6 from *Photobacterium damsela* (UniProt ID O66375) or a PdST6-like polypeptide consisting of amino acid residues 108 to 497 of UniProt ID O66375 having beta-galactoside alpha-2,6-sialyltransferase activity like SEQ ID NO 25, P-JT-ISH-224-ST6 from

Photobacterium sp. JT-ISH-224 (UniProt ID A8QYL1) or a P-JT-ISH-224-ST6-like polypeptide consisting of amino acid residues 18 to 514 of UniProt ID A8QYL1 having beta-galactoside alpha-2,6-sialyltransferase activity like SEQ ID NO 26 and/or an alpha-2,8-sialyltransferase like e.g. from *M. musculus* (UniProt ID Q64689). Constitutive transcriptional units of *PmNeuA* and the sialyltransferases can be delivered to the mutant strain either via genomic knock-in or via expression plasmids. If the mutant strains producing sialic acid and CMP-sialic acid were intended to make sialylated lactose structures, the strains were additionally modified with genomic knock-outs of the *E. coli* *LacZ*, *LacY* and *LacA* genes and with a genomic knock-in of a constitutive transcriptional unit for a lactose permease like e.g. the *E. coli* *LacY* with SEQ ID NO 15.

Alternatively, and/or additionally, sialic acid and/or sialylated oligosaccharide production can further be optimized in the mutant *E. coli* strains with a genomic knock-in of a constitutive transcriptional unit comprising a membrane transporter protein like e.g. a sialic acid transporter like e.g. nanT from *E. coli* K-12 MG1655 (UniProt ID P41036), nanT from *E. coli* O6:H1 (UniProt ID Q8FD59), nanT from *E. coli* O157:H7 (UniProt ID Q8X9G8) or nanT from *E. albertii* (UniProt ID B1EFH1) or a porter like e.g. EntS from *E. coli* (UniProt ID P24077), EntS from *Kluyvera ascorbata* (UniProt ID A0A378GQ13) or EntS from *Salmonella enterica subsp. arizonae* (UniProt ID A0A6Y2K4E8), MdfA from *Cronobacter muytjensii* (UniProt ID A0A2T7ANQ9), MdfA from *Citrobacter youngae* (UniProt ID D4BC23), MdfA from *E. coli* (UniProt ID P0AEY8), MdfA from *Yokenella regensburgei* (UniProt ID G9Z5F4), iceT from *E. coli* (UniProt ID A0A024L207), iceT from *Citrobacter youngae* (UniProt ID D4B8A6), SetA from *E. coli* (UniProt ID P31675), SetB from *E. coli* (UniProt ID P33026) or SetC from *E. coli* (UniProt ID P31436) or an ABC transporter like e.g. oppF from *E. coli* (UniProt ID P77737), ImrA from *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* (UniProt ID A0A1V0NEL4), or Blon_2475 from *Bifidobacterium longum* subsp. *infantis* (UniProt ID B7GPD4).

All mutant strains producing sialic acid, CMP-sialic acid and/or sialylated oligosaccharides could optionally be adapted for growth on sucrose via genomic knock-ins of constitutive transcriptional units containing a sucrose transporter like e.g. CscB from *E. coli* W with SEQ ID NO 01, a fructose kinase like e.g. Frk originating from *Z. mobilis* with SEQ ID NO 02 and a sucrose phosphorylase like e.g. BaSP originating from *B. adolescentis* with SEQ ID NO 03.

In an example to produce LN3 (GlcNAc-b1,3-Gal-b1,4-Glc) and oligosaccharides originating thereof comprising lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT), the mutant strain was derived from *E. coli* K12 MG1655 and modified with a knock-out of the *E. coli* *LacZ* and *nagB* genes and with a genomic knock-in of a constitutive transcriptional unit for a galactoside beta-1,3-N-acetylglucosaminyltransferase like e.g. LgtA from *N. meningitidis* with SEQ ID NO 27. For LNT or LNnT production, the mutant strain is further modified with constitutive transcriptional units for an N-acetylglucosamine beta-1,3-galactosyltransferase like e.g. WbgO from *E. coli* O55:H7 with SEQ ID NO 28 or an N-acetylglucosamine beta-1,4-galactosyltransferase like e.g. LgtB from *N. meningitidis* with SEQ ID NO 29, respectively, that can be delivered to the strain either via genomic knock-in or from an expression plasmid. Optionally, multiple

copies of the galactoside beta-1,3-N-acetylglucosaminyltransferase, the N-acetylglucosamine beta-1,3-galactosyltransferase and/or the N-acetylglucosamine beta-1,4-galactosyltransferase genes could be added to the mutant *E. coli* strains. Also, LNT and/or LNT production can be enhanced by improved UDP-GlcNAc production by modification of the strains with one or more genomic knock-ins of a constitutive transcriptional unit for an L-glutamine—D-fructose-6-phosphate aminotransferase like e.g. glmS*54 from *E. coli* with SEQ ID NO 19. In addition, the strains can optionally be modified for enhanced UDP-galactose production with genomic knock-outs of the *E. coli* *ushA*, *galT*, *ldhA* and *agp* genes. The mutant *E. coli* strains can also optionally be adapted with a genomic knock-in of a constitutive transcriptional unit for an UDP-glucose-4-epimerase like e.g. galE from *E. coli* with SEQ ID NO 30, a phosphoglucosamine mutase like e.g. glmM from *E. coli* with SEQ ID NO 42 and an N-acetylglucosamine-1-phosphate uridylyltransferase / glucosamine-1-phosphate acetyltransferase like e.g. glmU from *E. coli* with SEQ ID NO 43. The mutant strains could also optionally be adapted for growth on sucrose via genomic knock-ins of constitutive transcriptional units containing a sucrose transporter like e.g. CscB from *E. coli* W with SEQ ID NO 01, a fructose kinase like e.g. Frk originating from *Z. mobilis* with SEQ ID NO 02 and a sucrose phosphorylase like e.g. BaSP originating from *B. adolescentis* with SEQ ID NO 03.

Alternatively, and/or additionally, production of LN3, LNT, LNT and oligosaccharides derived thereof can further be optimized in the mutant *E. coli* strains with a genomic knock-in of a constitutive transcriptional unit comprising a membrane transporter protein like e.g. MdfA from *Cronobacter muytjensii* (UniProt ID A0A2T7ANQ9), MdfA from *Citrobacter youngae* (UniProt ID D4BC23), MdfA from *E. coli* (UniProt ID P0AEY8), MdfA from *Yokenella regensburgei* (UniProt ID G9Z5F4), iceT from *E. coli* (UniProt ID A0A024L207) or iceT from *Citrobacter youngae* (UniProt ID D4B8A6).

Preferably but not necessarily, the glycosyltransferases, the proteins involved in nucleotide-activated sugar synthesis and/or membrane transporter proteins were N- and/or C-terminally fused to a solubility enhancer tag like e.g. a SUMO-tag, an MBP-tag, His, FLAG, Strep-II, Halo-tag, NusA, thioredoxin, GST and/or the Fh8-tag to enhance their solubility (Costa et al., Front. Microbiol. 2014, <https://doi.org/10.3389/fmicb.2014.00063>; Fox et al., Protein Sci. 2001, 10(3), 622-630; Jia and Jeon, Open Biol. 2016, 6: 160196).

Optionally, the mutant *E. coli* strains were modified with a genomic knock-in of a constitutive transcriptional unit encoding a chaperone protein like e.g. DnaK, DnaJ, GrpE, or the GroEL/ES chaperonin system (Baneyx F., Palumbo J.L. (2003) Improving Heterologous Protein Folding via Molecular Chaperone and Foldase Co-Expression. In: Vaillancourt P.E. (eds) *E. coli* Gene Expression Protocols. Methods in Molecular Biology™, vol 205. Humana Press).

Optionally, the mutant *E. coli* strains are modified to create a glycominimized *E. coli* strain comprising genomic knock-out of any one or more of non-essential glycosyltransferase genes comprising *pgaC*, *pgaD*, *rfe*, *rffT*, *rffM*, *bcsA*, *bcsB*, *bcsC*, *wcaA*, *wcaC*, *wcaE*, *wcaI*, *wcaJ*, *wcaL*, *waaH*, *waaF*, *waaC*, *waaU*, *waaZ*, *waaJ*, *waaO*, *waaB*, *waaS*, *waaG*, *waaQ*, *wbbI*, *arnC*, *arnT*, *yfdH*, *wbbK*, *opgG*, *opgH*, *ycjM*, *glgA*, *glgB*, *malQ*,

otsA and yaiP.

All constitutive promoters, UTRs and terminator sequences originated from the libraries described by Mutalik et al. (Nat. Methods 2013, No. 10, 354-360) and Cambray et al. (Nucleic Acids Res. 2013, 41(9), 5139-5148): the genes were expressed using promoters MutalikP5 ("PROM0005_MutalikP5") and apFAB82 ("PROM0050_apFAB82") as described by Mutalik et al. (Nat. Methods 2013, No. 10, 354-360), UTRs used comprised GalE_BCD12 ("UTR0010_GalE_BCD12") and GalE_LeuAB ("UTR0014_GalE_LeuAB") as described by Mutalik et al. (Nat. Methods 2013, No. 10, 354-360), and terminator sequence used was ilvGEDA ("TER0007_ilvGEDA") as described by Cambray et al. (Nucleic Acids Res. 2013, 41(9), 5139-5148). All genes were ordered synthetically at Twist Bioscience (twistbioscience.com) or IDT (eu.idtdna.com) and the codon usage was adapted using the tools of the supplier. The SEQ ID NOs described in present invention are summarized in Table 1.

All strains were stored in cryovials at -80°C (overnight LB culture mixed in a 1:1 ratio with 70% glycerol).

Table 1: Overview of SEQ ID NOs described in the present invention

SEQ ID NO	Name / identifier	Organism	Origin	Country of origin of digital sequence information
01	CscB	Escherichia coli W	Synthetic	USA
02	Frk	Zymomonas mobilis	Synthetic	United Kingdom
03	BaSP	Bifidobacterium adolescentis	Synthetic	Germany
04	HpFutC	Helicobacter pylori UA1234	Synthetic	United Kingdom
05	HpFucT	Helicobacter pylori UA1234	Synthetic	United Kingdom
06	Mutant HpFucT	Helicobacter pylori UA1234	Synthetic	United Kingdom
07	thyA	Escherichia coli K-12 MG1655	Synthetic	USA
08	manA	Escherichia coli K-12 MG1655	Synthetic	USA
09	manB	Escherichia coli K-12 MG1655	Synthetic	USA
10	manC	Escherichia coli K-12 MG1655	Synthetic	USA
11	gmd	Escherichia coli K-12 MG1655	Synthetic	USA
12	fcl	Escherichia coli K-12 MG1655	Synthetic	USA
13	fucP	Escherichia coli K-12 MG1655	Synthetic	USA

14	fkp	Bacteroides fragilis	Synthetic	United Kingdom
15	LacY	Escherichia coli K-12 MG1655	Synthetic	USA
16	GNA1	Saccharomyces cerevisiae	Synthetic	USA
17	AGE	Bacteroides ovatus	Synthetic	USA
18	neuB	Neisseria meningitidis	Synthetic	United Kingdom
19	glmS*54	Escherichia coli K-12 MG1655	Synthetic	USA
20	phosphatase	Escherichia coli K-12 MG1655	Synthetic	USA
21	neuC	Campylobacter jejuni	Synthetic	USA
22	neuA	Pasteurella multocida	Synthetic	USA
23	alpha-2,3-sialyltransferase	Pasteurella multocida	Synthetic	USA
24	alpha-2,3-sialyltransferase	Neisseria meningitidis	Synthetic	United Kingdom
25	alpha-2,6-sialyltransferase	Photobacterium damsela	Synthetic	Japan
26	alpha-2,6-sialyltransferase	Photobacterium sp. JT-ISH-224	Synthetic	Japan
27	LgtA	Neisseria meningitidis	Synthetic	United Kingdom
28	WbgO	Escherichia coli O55:H7	Synthetic	Germany
29	LgtB	Neisseria meningitidis MC58	Synthetic	United Kingdom
30	gale	Escherichia coli K-12 MG1655	Synthetic	USA
31	Lac12	Kluyveromyces lactis	Synthetic	USA
32	GCNT2	Homo sapiens	Synthetic	Unknown
33	alpha-1,3-galactosyltransferase	Bos taurus	Synthetic	Unknown
34	WbpP	Pseudomonas aeruginosa	Synthetic	USA
35	lgtD	Haemophilus influenzae	Synthetic	Ireland
36	lgtC	Neisseria gonorrhoeae	Synthetic	Japan
37	WbnI	Escherichia coli K-12 MG1655	Synthetic	USA
38	BgtA	Helicobacter mustelae	Synthetic	Unknown
39	Mutant a1,3/4 fucosidase	Bifidobacterium longum subsp.	Synthetic	USA

		infantis		
40	fucT54	Sideroxydans lithotrophicus ES-1	Synthetic	USA
41	WbdO	Salmonella enterica subsp. salamae serovar Greenside	Synthetic	United Kingdom
42	glmM	Escherichia coli K-12 MG1655	Synthetic	USA
43	glmU	Escherichia coli K-12 MG1655	Synthetic	USA

Cultivation conditions

A preculture of 96-well microtiter plate experiments was started from a cryovial, in 150 µL LB and was incubated overnight at 37 °C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96well square microtiter plate, with 400 µL minimal medium by diluting 400x. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer. To measure sugar concentrations at the end of the cultivation experiment whole broth samples were taken from each well by boiling the culture broth for 15 min at 60°C before spinning down the cells (= average of intra- and extracellular sugar concentrations).

A preculture for the bioreactor was started from an entire 1 mL cryovial of a certain strain, inoculated in 250 mL or 500 mL minimal medium in a 1 L or 2.5 L shake flask and incubated for 24 h at 37°C on an orbital shaker at 200 rpm. A 5 L bioreactor was then inoculated (250 mL inoculum in 2 L batch medium); the process was controlled by MFCS control software (Sartorius Stedim Biotech, Melsungen, Germany). Culturing condition were set to 37 °C, and maximal stirring; pressure gas flow rates were dependent on the strain and bioreactor. The pH was controlled at 6.8 using 0.5 M H₂SO₄ and 20% NH₄OH. The exhaust gas was cooled. 10% solution of silicone antifoaming agent was added when foaming raised during the fermentation.

Optical density

Cell density of the cultures was frequently monitored by measuring optical density at 600 nm (Implen Nanophotometer NP80, Westburg, Belgium or with a Spark 10M microplate reader, Tecan, Switzerland).

Analytical analysis

Standards such as but not limited to sucrose, lactose, LacNAc, lacto-N-biose (LNB), fucosylated LacNAc (2'FLacNAc, 3-FLacNAc), sialylated LacNAc, (3'SLacNAc, 6'SLacNAc), fucosylated LNB (2'FLNB, 4'FLNB), lacto-N-triose II (LN3), lacto-N-tetraose (LNT), lacto-N-neo-tetraose (LNnT), LNFP-I, LNFP-II, LNFP-III, LNFP-V, LSTa, LSTc and LSTd were purchased from Carbosynth (UK), Elicityl (France) and IsoSep (Sweden). Other compounds were analyzed with in-house made standards.

Neutral mammalian milk oligosaccharides were analyzed on a Waters Acquity H-class UPLC with Evaporative Light Scattering Detector (ELSD) or a Refractive Index (RI) detection. A volume of 0.7 μL sample was injected on a Waters Acquity UPLC BEH Amide column (2.1 x 100 mm; 130 $\text{\AA}$; 1.7 μm) column with an Acquity UPLC BEH Amide VanGuard column, 130 $\text{\AA}$, 2.1 x 5 mm. The column temperature was 50 °C. The mobile phase consisted of a $\frac{1}{4}$ water and $\frac{3}{4}$ acetonitrile solution to which 0.2 % triethylamine was added. The method was isocratic with a flow of 0.130 mL/min. The ELS detector had a drift tube temperature of 50 °C and the N₂ gas pressure was 50 psi, the gain 200 and the data rate 10 pps. The temperature of the RI detector was set at 35 °C.

Sialylated mammalian milk oligosaccharides were analyzed on a Waters Acquity H-class UPLC with Refractive Index (RI) detection. A volume of 0.5 μL sample was injected on a Waters Acquity UPLC BEH Amide column (2.1 x 100 mm; 130 $\text{\AA}$; 1.7 μm). The column temperature was 50 °C. The mobile phase consisted of a mixture of 70 % acetonitrile, 26 % ammonium acetate buffer (150 mM) and 4 % methanol to which 0.05 % pyrrolidine was added. The method was isocratic with a flow of 0.150 mL/min. The temperature of the RI detector was set at 35 °C.

Both neutral and sialylated sugars were analyzed on a Waters Acquity H-class UPLC with Refractive Index (RI) detection. A volume of 0.5 μL sample was injected on a Waters Acquity UPLC BEH Amide column (2.1 x 100 mm; 130 $\text{\AA}$; 1.7 μm). The column temperature was 50 °C. The mobile phase consisted of a mixture of 72% acetonitrile and 28% ammonium acetate buffer (100 mM) to which 0.1% triethylamine was added. The method was isocratic with a flow of 0.260 mL/min. The temperature of the RI detector was set at 35 °C.

For analysis on a mass spectrometer, a Waters Xevo TQ-MS with Electron Spray Ionisation (ESI) was used with a desolvation temperature of 450 °C, a nitrogen desolvation gas flow of 650 L/h and a cone voltage of 20 V. The MS was operated in selected ion monitoring (SIM) in negative mode for all mammalian milk oligosaccharides. Separation was performed on a Waters Acquity UPLC with a Thermo Hypercarb column (2.1 x 100 mm; 3 μm) on 35 °C. A gradient was used wherein eluent A was ultrapure water with 0.1 % formic acid and wherein eluent B was acetonitrile with 0.1 % formic acid. The mammalian milk oligosaccharides were separated in 55 min using the following gradient: an initial increase from 2 to 12 % of eluent B over 21 min, a second increase from 12 to 40 % of eluent B over 11 min and a third increase from 40 to 100 % of eluent B over 5 min. As a washing step 100 % of eluent B was used for 5 min. For column equilibration, the initial condition of 2 % of eluent B was restored in 1 min and maintained for 12 min.

Both neutral and sialylated sugars at low concentrations (below 50 mg/L) were analyzed on a Dionex HPAEC system with pulsed amperometric detection (PAD). A volume of 5 μL of sample was injected on a Dionex CarboPac PA200 column 4 x 250 mm with a Dionex CarboPac PA200 guard column 4 x 50 mm. The column temperature was set to 30 °C. A gradient was used wherein eluent A was deionized water, wherein eluent B was 200 mM Sodium hydroxide and wherein eluent C was 500 mM Sodium acetate. The

oligosaccharides were separated in 60 min while maintaining a constant ratio of 25 % of eluent B using the following gradient: an initial isocratic step maintained for 10 min of 75 % of eluent A, an initial increase from 0 to 4 % of eluent C over 8 min, a second isocratic step maintained for 6 min of 71 % of eluent A and 4 % of eluent C, a second increase from 4 to 12 % of eluent C over 2.6 min, a third isocratic step maintained for 3.4 min of 63 % of eluent A and 12 % of eluent C and a third increase from 12 to 48 % of eluent C over 5 min. As a washing step 48 % of eluent C was used for 3 min. For column equilibration, the initial condition of 75 % of eluent A and 0 % of eluent C was restored in 1 min and maintained for 11 min. The applied flow was 0.5 mL/min.

10 Example 2. Materials and Methods *Saccharomyces cerevisiae*

Media

Strains were grown on Synthetic Defined yeast medium with Complete Supplement Mixture (SD CSM) or CSM drop-out (SD CSM-Ura, SD CSM-Trp, SD CSM-His) containing 6.7 g/L Yeast Nitrogen Base without amino acids (YNB w/o AA, Difco), 20 g/L agar (Difco) (solid cultures), 22 g/L glucose monohydrate or 20 g/L lactose and 0.79 g/L CSM or 0.77 g/L CSM-Ura, 0.77 g/L CSM-Trp, or 0.77 g/L CSM-His (MP Biomedicals).

Strains

S. cerevisiae BY4742 created by Brachmann *et al.* (Yeast (1998) 14:115-32) was used, available in the Euroscarf culture collection. All mutant strains were created by homologous recombination or plasmid transformation using the method of Gietz (Yeast 11:355-360, 1995).

Plasmids

In an example to produce GDP-fucose, the yeast expression plasmid p2a_2 μ _Fuc (Chan 2013, Plasmid 70, 2-17) was used for expression of foreign genes in *S. cerevisiae*. This plasmid contained an ampicillin resistance gene and a bacterial origin of replication to allow for selection and maintenance in *E. coli* and the 2 μ yeast ori and the Ura3 selection marker for selection and maintenance in yeast. This plasmid further contained constitutive transcriptional units for a lactose permease like e.g. LAC12 from *Kluyveromyces lactis* with SEQ ID NO 31, a GDP-mannose 4,6-dehydratase like e.g. gmd from *E. coli* with SEQ ID NO 11 and a GDP-L-fucose synthase like e.g. fcl from *E. coli* with SEQ ID NO 12. In another example the yeast expression plasmid p2a_2 μ _Fuc2 can be used as an alternative expression plasmid of the p2a_2 μ _Fuc plasmid comprising next to the ampicillin resistance gene, the bacterial ori, the 2 μ yeast ori and the Ura3 selection marker constitutive transcriptional units for a lactose permease like e.g. LAC12 from *K. lactis* with SEQ ID NO 31, a fucose permease like e.g. fucP from *E. coli* with SEQ ID NO 13 and a bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase like e.g. fkp from *B. fragilis* with SEQ NO ID 14. To further produce fucosylated oligosaccharides, the p2a_2 μ _Fuc and its variant the

p2a_2μ_Fuc2, additionally contains a constitutive transcriptional unit for an alpha-1,2-fucosyltransferase like e.g. HpFutC from *H. pylori* with SEQ ID NO 04 and/or an alpha-1,3-fucosyltransferase like e.g. HpFucT from *H. pylori* with SEQ ID NO 05.

In an example to produce sialic acid and CMP-sialic acid, a yeast expression plasmid was derived from the pRS420-plasmid series (Christianson et al., 1992, Gene 110: 119-122) containing the TRP1 selection marker and constitutive transcriptional units for an L-glutamine—D-fructose-6-phosphate aminotransferase like e.g. the mutant glmS*54 from *E. coli* with SEQ ID NO 19, a phosphatase like e.g. YqaB from *E. coli* with SEQ ID NO 20 or the *E. coli* genes comprising aphA, Cof, HisB, OtsB, SurE, Yaed, YcjU, YedP, YfbT, YidA, YigB, YihX, YniC, YrbL, AppA, Gph, SerB, YbhA, YbiV, YbjL, Yfb, YieH, YjgL, YjjG, YrfG and YbiU or PsMupP from *Pseudomonas putida*, ScDOG1 from *S. cerevisiae* and BsAraL from *Bacillus subtilis* as described in WO18122225, an N-acetylglucosamine 2-epimerase like e.g. AGE from *B. ovatus* with SEQ ID NO 17, an N-acetylneuraminate synthase like e.g. NeuB from *N. meningitidis* with SEQ ID NO 18 and an N-acetylneuraminate cytidyltransferase like e.g. NeuA from *P. multocida* with SEQ ID NO 22. Optionally, a constitutive transcriptional unit for a glucosamine 6-phosphate N-acetyltransferase like e.g. GNA1 from *S. cerevisiae* with SEQ ID NO 16 was added as well. To produce sialylated oligosaccharides, the plasmid further comprised constitutive transcriptional units for a lactose permease like e.g. LAC12 from *K. lactis* with SEQ ID NO 31, and a beta-galactoside alpha-2,3-sialyltransferase like e.g. PmultST3 from *P. multocida* (UniProt ID Q9CLP3) or a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity like SEQ ID NO 23, NmeniST3 from *N. meningitidis* (SEQ ID NO 24) or PmultST2 from *P. multocida* subsp. *multocida* str. Pm70 (GenBank NO. AAK02592.1), a beta-galactoside alpha-2,6-sialyltransferase like e.g. PdST6 from *Photobacterium damsela* (UniProt ID O66375) or a PdST6-like polypeptide consisting of amino acid residues 108 to 497 of UniProt ID O66375 having beta-galactoside alpha-2,6-sialyltransferase activity like SEQ ID NO 25, P-JT-ISH-224-ST6 from *Photobacterium* sp. JT-ISH-224 (UniProt ID A8QYL1) or a P-JT-ISH-224-ST6-like polypeptide consisting of amino acid residues 18 to 514 of UniProt ID A8QYL1 having beta-galactoside alpha-2,6-sialyltransferase activity like SEQ ID NO 26 and/or an alpha-2,8-sialyltransferase like e.g. from *M. musculus* (UniProt ID Q64689).

In an example to produce UDP-galactose, a yeast expression plasmid was derived from the pRS420-plasmid series (Christianson et al., 1992, Gene 110: 119-122) containing the HIS3 selection marker and a constitutive transcriptional unit for an UDP-glucose-4-epimerase like e.g. galE from *E. coli* with SEQ ID NO 30. To produce LN3 this plasmid was further modified with constitutive transcriptional units for a lactose permease like e.g. LAC12 from *K. lactis* with SEQ ID NO 31, a galactoside beta-1,3-N-acetylglucosaminyltransferase (lgtA) from *N. meningitidis* with SEQ ID NO 27. To produce LN3-derived oligosaccharides like LNT or LNTnT, the plasmid was further modified with constitutive transcriptional units for an N-acetylglucosamine beta-1,3-galactosyltransferase like e.g. WbgO from *E. coli* O55:H7 with SEQ ID NO 28 or an N-acetylglucosamine beta-1,4-galactosyltransferase like e.g. lgtB from *N. meningitidis* with

SEQ ID NO 29, respectively.

Preferably but not necessarily, any one or more of the glycosyltransferases, the proteins involved in nucleotide-activated sugar synthesis and/or membrane transporter proteins were N- and/or C-terminally fused to a SUMOstar tag (e.g. obtained from pYSUMOstar, Life Sensors, Malvern, PA) to enhance their solubility.

Optionally, the mutant yeast strains were modified with a genomic knock-in of a constitutive transcriptional unit encoding a chaperone protein like e.g. Hsp31, Hsp32, Hsp33, Sno4, Kar2, Ssb1, Sse1, Sse2, Ssa1, Ssa2, Ssa3, Ssa4, Ssb2, Ecm10, Ssc1, Ssq1, Ssz1, Lhs1, Hsp82, Hsc82, Hsp78, Hsp104, Tcp1, Cct4, Cct8, Cct2, Cct3, Cct5, Cct6, or Cct7 (Gong et al., 2009, Mol. Syst. Biol. 5: 275).

Plasmids were maintained in the host *E. coli* DH5alpha (F⁻, phi80d*lacZ*deltaM15, delta(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, lambda⁻, *thi-1*, *gyrA96*, *relA1*) bought from Invitrogen.

Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9, IDT or Twist Bioscience. Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Cultivations conditions

In general, yeast strains were initially grown on SD CSM plates to obtain single colonies. These plates were grown for 2-3 days at 30 °C. Starting from a single colony, a preculture was grown over night in 5 mL at 30 °C, shaking at 200 rpm. Subsequent 125 mL shake flask experiments were inoculated with 2% of this preculture, in 25 mL media. These shake flasks were incubated at 30 °C with an orbital shaking of 200 rpm.

Gene expression promoters

Genes were expressed using synthetic constitutive promoters, as described by Blazeck (Biotechnology and Bioengineering, Vol. 109, No. 11, 2012).

Example 3. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL with a modified *E. coli* host expressing an alpha-1,2-fucosyltransferase and an alpha-1,3-fucosyltransferase from two compatible plasmids

An *E. coli* K12 strain modified for GDP-fucose production as described in Example 1 was sequentially transformed with a first plasmid expressing a constitutive transcriptional unit for the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and a second compatible plasmid expressing a constitutive transcriptional unit for the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. The novel strain was

evaluated in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contained sucrose and lactose. Each strain was grown in four biological replicates in a 96-well plate. After 72h of incubation, the culture broth was harvested, and the sugar mixtures were analyzed on UPLC. The experiment demonstrated the novel strain produced an oligosaccharide mixture comprising 2'FL, 3-FL and difucosylated lactose (DiFL) in whole broth samples.

Example 4. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL with a modified *E. coli* host expressing an alpha-1,2-fucosyltransferase and an alpha-1,3-fucosyltransferase from one plasmid

An *E. coli* strain modified for GDP-fucose production as described in Example 1 was further transformed with one expression plasmid having two constitutive transcriptional units, including one for the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and another one for the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. The novel strain was evaluated in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contained sucrose and lactose. Each strain was grown in four biological replicates in a 96-well plate. After 72h of incubation, the culture broth was harvested, and the sugar mixtures were analyzed on UPLC. The experiment demonstrated the novel strain produced an oligosaccharide mixture comprising 2'FL, 3-FL and difucosylated lactose (DiFL) in whole broth samples.

Example 5. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL with a modified *E. coli* host in fed-batch fermentations

Mutant *E. coli* strains as described in Examples 3 and 4 were further evaluated in a fed-batch fermentation process. Fed-batch fermentations at bioreactor scale were performed as described in Example 1. In these examples, sucrose was used as a carbon source and lactose was added in the batch medium as a precursor. Regular broth samples were taken and the production of 2'FL, 3-FL and DiFL was measured using UPLC as described in Example 1. The experiment demonstrated that broth samples taken at the end of batch phase comprised an oligosaccharide mixture of 2'FL and 3-FL together with unmodified lactose, whereas broth samples taken at the end of the fed-batch phase comprised an oligosaccharide mixture of 2'FL, 3-FL and DiFL. As the ratios of lactose, 2'FL, 3-FL and DiFL changed over time during fed-batch, they could be manipulated during the fermentation process by discontinuation of the fermentation process at a desired time in fed-batch phase.

Example 6. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 2'FLacNAc, 3-FLacNAc and Di-FLacNAc with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, was further transformed with an expression plasmid having two constitutive transcriptional units, one to express the

H. pylori alpha-1,2-fucosyltransferase with SEQ ID NO 04 and the other one to express the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. Since lactose and N-acetyllactosamine (LacNAc, Gal-b1,4-GlcNAc) are suitable acceptors for both *H. pylori* fucosyltransferases, the novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL and fucosylated LacNAc (i.e. 2'FLacNAc and 3-FLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose and LacNAc as precursors. Since the enzyme with SEQ ID NO 04 also shows fucosyltransferase activity on 2'FL and the enzyme with SEQ ID NO 05 also shows fucosyltransferase activity on 2'FLacNAc the novel strain is also evaluated for production of DiFL and Di-FLacNAc in said oligosaccharide mixture.

Example 7. Production of a mammalian milk oligosaccharide mixture comprising 2'FLacNAc, 3-FLacNAc and Di-FLacNAc with a modified *E. coli* host expressing different fucosyltransferases

An *E. coli* K-12 MG1655 strain optimized for GDP-fucose production as described in Example 1 is further modified for the production of GlcNAc and LacNAc with a knock-out of the *E. coli* N-acetylglucosamine-6-phosphate deacetylase (*nagA*) gene and the *E. coli* glucosamine-6-phosphate deaminase (*nagB*) gene together with genomic knock-ins of constitutive transcriptional units for the N-acetylglucosamine β 1,4-galactosyltransferase (LgtB) of *N. meningitidis* with SEQ ID NO 29, the mutant L-glutamine-D-fructose-6-phosphate aminotransferase (glmS*54) from *E. coli* with SEQ ID NO 19, and the glucosamine 6-phosphate N-acetyltransferase (GNA1) of *S. cerevisiae* with SEQ ID NO 16. In a next step, the novel strain is additionally transformed with two compatible expression plasmids wherein the first plasmid contains a constitutive transcriptional unit for the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and the second compatible plasmid contains a constitutive transcriptional unit for the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. Alternatively, the novel strain can be transformed with one expression plasmid containing a constitutive transcriptional unit for both *H. pylori* fucosyltransferases with SEQ ID NO 04 and 05. All novel strains are evaluated for production of a mixture of 2'-fucosylated LacNAc (2'FLacNAc), 3-fucosylated LacNAc (3-FLacNAc) and di-fucosylated LacNAc (Di-FLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the cultivation contains sucrose as carbon source.

Example 8. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 2'FLNB, 4-FLNB and Di-FLNB with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, is further transformed with an expression plasmid having two constitutive transcriptional units, one to express the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and one to express the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. Since lactose and lacto-N-biose (LNB, Gal-b1,3-GlcNAc) are suitable acceptors for both *H. pylori* fucosyltransferases, the novel strain is evaluated for production of an

oligosaccharide mixture comprising 2'FL, 3-FL and fucosylated LNB (i.e. 2'FLNB and 4-FLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose and LNB as precursors. Since the enzyme with SEQ ID NO 04 also shows fucosyltransferase activity on 2'FL and the enzyme with SEQ ID NO 05 also shows fucosyltransferase activity on 2'FLNB the novel strain is also evaluated for production of DiFL and Di-FLNB in said oligosaccharide mixture.

Example 9. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 2'FLNB, 4-FLNB and Di-FLNB with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1 is further adapted for intracellular lactose synthesis by genomic knock-outs of *lacZ*, *glk* and the *galETKM* operon, together with genomic knock-ins of constitutive transcriptional units for *IgtB* from *N. meningitidis* with SEQ ID NO 29 and the UDP-glucose 4-epimerase (*galE*) from *E. coli* with SEQ ID NO 30. In a next step, the mutant *E. coli* strain was transformed with an expression plasmid having two constitutive transcriptional units, one to express the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and one to express the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL and fucosylated LNB (i.e. 2'FLNB, 4-FLNB and DiFLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and LNB as precursor.

Example 10. Production of a mammalian milk oligosaccharide mixture comprising 2'FLNB, 4-FLNB and di-fucosylated LNB with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain optimized for GDP-fucose production as exemplified in Example 1, is further modified for the production of GlcNAc and LNB with a knock-out of the *E. coli* *nagA* and *nagB* genes, together with genomic knock-ins of constitutive transcriptional units for the N-acetylglucosamine β 1,3-galactosyltransferase (*WbgO*) from *E. coli* O55:H7 with SEQ ID NO 28, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, and *GNA1* from *S. cerevisiae* with SEQ ID NO 16. In a next step, the novel strain is additionally transformed with two compatible expression plasmids, one plasmid containing a constitutive transcriptional unit for the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and the other plasmid containing a constitutive transcriptional unit for the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'-fucosylated LNB (2'FLNB), 4-fucosylated LNB (4-FLNB) and di-fucosylated LNB (Di-FLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the cultivation contains sucrose as carbon source.

Example 11. Production of a mammalian milk oligosaccharide mixture comprising 2'FLNB, 4-FLNB and di-fucosylated LNB with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain optimized for GDP-fucose production as exemplified in Example 1, is further modified for the production of GlcNAc and LNB with a knock-out of the *E. coli* *nagA* and *nagB* genes, together with genomic knock-ins of constitutive transcriptional units for WbgO from *E. coli* O55:H7 with SEQ ID NO 28, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, and GNA1 from *S. cerevisiae* with SEQ ID NO 16. In a next step, the novel strain is additionally transformed with one expression plasmid containing a constitutive transcriptional unit for the *H. pylori* alpha-1,2-fucosyltransferase with SEQ ID NO 04 and a constitutive transcriptional unit for the *H. pylori* alpha-1,3-fucosyltransferase with SEQ ID NO 05.

The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'-fucosylated LNB (2'FLNB), 4-fucosylated LNB (4-FLNB) and di-fucosylated LNB (Di-FLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the cultivation contains sucrose as carbon source.

Example 12. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, 2'FLNB and Gal-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, is transformed with an expression plasmid having constitutive transcriptional units for the alpha-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and the alpha-1,3-galactosyltransferase WbnI from *E. coli* with SEQ ID NO 37.

Since lactose and LNB are suitable acceptors for the *H. pylori* alpha-1,2-fucosyltransferase the novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL and 2'-fucosylated LNB (2'FLNB) in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose and LNB as precursors. In this experiment the novel strain is additionally evaluated for production of Gal-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc in the oligosaccharide mixture since 2'FLNB is an acceptor of the *E. coli* alpha-1,3-galactosyltransferase.

Example 13. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, 2'FLNB and GalNAc-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, is transformed with an expression plasmid having constitutive transcriptional units for the alpha-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and the alpha-1,3-N-acetylgalactosaminyltransferase BgtA from *H. mustelae* with SEQ ID NO 38. Since lactose and LNB are suitable acceptors for the *H. pylori* alpha-1,2-fucosyltransferase the novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL and 2'-fucosylated LNB (2'FLNB) in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose and LNB as precursors. In this experiment the novel strain is additionally evaluated for production of GalNAc-

a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc in the oligosaccharide mixture since 2'FLNB is an acceptor of the *H. mustelae* alpha-1,3-N-acetylgalactosaminyltransferase.

Example 14. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, 2'FLNB and Gal-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, is further modified for the production of GlcNAc and LNB with a knock-out of the *E. coli* *nagA* and *nagB* genes, together with genomic knock-ins of constitutive transcriptional units for the N-acetylglucosamine β 1,3-galactosyltransferase (WbgO) from *E. coli* O55:H7 with SEQ ID NO 28, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, and GNA1 from *S. cerevisiae* with SEQ ID NO 16. In a next step, the novel strain is transformed with an expression plasmid containing constitutive transcriptional units for the alpha-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and the alpha-1,3-galactosyltransferase Wbni from *E. coli* with SEQ ID NO 37. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, 2'-fucosylated LNB and Gal-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 15. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, 2'FLNB and GalNAc-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc with a modified *E. coli* host

An *E. coli* strain modified for the production of GDP-fucose as described in Example 1, is further modified for the production of GlcNAc and LNB with a knock-out of the *E. coli* *nagA* and *nagB* genes, together with genomic knock-ins of constitutive transcriptional units for WbgO from *E. coli* O55:H7 with SEQ ID NO 28, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, and GNA1 from *S. cerevisiae* with SEQ ID NO 16. In a next step, the novel strain is transformed with an expression plasmid containing constitutive transcriptional units for the alpha-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and the alpha-1,3-N-acetylgalactosaminyltransferase BgtA from *H. mustelae* with SEQ ID NO 38. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, 2'-fucosylated LNB and GalNAc-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 16. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc and 6'-sialylated LacNAc with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain modified with a genomic knock-in of a constitutive transcriptional unit for the N-acetylneuraminyltransferase (*neuA*) from *P. multocida* with SEQ ID NO 22 and containing a knock-out of the *E. coli* *lacZ* gene is further transformed with an expression plasmid containing

constitutive transcriptional units for the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc (3'SLacNAc) and 6'-sialylated (6'SLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and sialic acid, lactose and LacNAc as precursors.

Example 17. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc and 6'-sialylated LacNAc with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain modified with a genomic knock-in of a constitutive transcriptional unit for the N-acetylneuraminase cytidyltransferase (*neuA*) from *P. multocida* with SEQ ID NO 22 is further mutated with a genomic knock-out of the *E. coli* *nagA*, *nagB* and *lacZ* genes together with genomic knock-ins of constitutive transcriptional units for the mutant *glmS**54 with SEQ ID NO 19 from *E. coli*, *GNA1* with SEQ ID NO 16 from *S. cerevisiae*, the phosphatase *yqaB* from *E. coli* with SEQ ID NO 20 and *LgtB* with SEQ ID NO 29 from *N. meningitidis*. In a next step, the novel strain is transformed with an expression plasmid containing constitutive transcriptional units for the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc (3'SLacNAc) and 6'-sialylated LacNAc (6'SLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and sialic acid and lactose as precursors.

Example 18. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc and 6'-sialylated LacNAc with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further modified with a knock-out of the *E. coli* *lacZ* gene and transformed with an expression plasmid consisting of constitutive transcriptional units for *neuA* from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc (3'SLacNAc) and 6'-sialylated LacNAc (6'SLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose and LacNAc as precursors.

Example 19. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc and 6'-sialylated LacNAc with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further mutated with a genomic knock-out of the *E. coli lacZ* gene together with a genomic knock-in of a constitutive transcriptional unit for LgtB with SEQ ID NO 29 from *N. meningitidis* to produce LacNAc, and transformed with an expression plasmid containing constitutive transcriptional units for neuA from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LacNAc (3'SLacNAc) and 6'-sialylated LacNAc (6'SLacNAc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

Example 20. Production of sialylated LacNAc and poly-LacNAc structures with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further mutated with a genomic knock-in of a constitutive transcriptional unit for LgtB with SEQ ID NO 29 from *N. meningitidis* to produce LacNAc, and transformed with an expression plasmid containing constitutive transcriptional units for neuA from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. In a next step, the mutant strain is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from *N. meningitidis* with SEQ ID NO 27. The novel strain is evaluated for production of LacNAc and an oligosaccharide mixture comprising poly-LacNAc structures, i.e. (Gal-b1,4-GlcNAc)_n which are built of repeated N-acetylglucosamine units that are beta1,3-linked to each other by alternate activity of both the transferases LgtB with SEQ ID NO 29 and LgtA with SEQ ID NO 27, together with 3'-sialylated LacNAc and 6'-sialylated LacNAc, and sialylated poly-LacNAc structures in which the Gal residue is sialylated, in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and wherein no precursor needs to be supplied to the cultivation.

Example 21. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB and 6'-sialylated LNB with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified with a genomic knock-in of a constitutive transcriptional unit for neuA from *P. multocida* with SEQ ID NO 22 and containing a knock-out of the *E. coli lacZ* gene is further transformed with an expression plasmid containing constitutive transcriptional units for the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture

comprising 3'SL, 6'SL, 3'-sialylated LNB (3'SLNB) and 6'-sialylated LNB (6'SLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and sialic acid, lactose and LNB as precursors.

Example 22. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB and 6'-sialylated LNB with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified with a genomic knock-in of a constitutive transcriptional unit for neuA from *P. multocida* with SEQ ID NO 22 is further mutated with a genomic knock-out of the *E. coli* nagA, nagB and lacZ genes together with genomic knock-ins of constitutive transcriptional units for the mutant glmS*54 with SEQ ID NO 19 from *E. coli*, GNA1 with SEQ ID NO 16 from *S. cerevisiae*, the phosphatase yqaB from *E. coli* with SEQ ID NO 20 and WbgO with SEQ ID NO 28 from *E. coli* O55:H7 to produce LNB. In a next step, the novel strain is transformed with an expression plasmid containing constitutive transcriptional units for the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB (3'SLNB) and 6'-sialylated LNB (6'SLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and sialic acid and lactose as precursors.

Example 23. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB and 6'-sialylated LNB with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further modified with a knock-out of the *E. coli* lacZ gene and transformed with an expression plasmid consisting of constitutive transcriptional units for neuA from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB (3'SLNB) and 6'-sialylated LNB (6'SLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose and LNB as precursors.

Example 24. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB and 6'-sialylated LNB with a modified E. coli host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further mutated with a genomic knock-out of the *E. coli* lacZ gene together with a genomic knock-in of a constitutive transcriptional unit for WbgO with SEQ ID NO 28 from *E. coli* O55:H7 to produce LNB, and transformed with an expression plasmid containing constitutive transcriptional units for neuA from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-

sialyltransferase from *P. damselae* with SEQ ID NO 25. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'SL, 6'SL, 3'-sialylated LNB (3'SLNB) and 6'-sialylated LNB (6'SLNB) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

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Example 25. Production of sialylated LNB with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain modified to produce sialic acid as described in Example 1 is further mutated with a genomic knock-in of a constitutive transcriptional unit for WbgO with SEQ ID NO 28 from *E. coli* O55:H7 to produce LNB, and transformed with an expression plasmid containing constitutive
10 transcriptional units for neuA from *P. multocida* with SEQ ID NO 22, the alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the alpha-2,6-sialyltransferase from *P. damselae* with SEQ ID NO 25. The novel strain is evaluated for production of LNB together with 3'-sialylated LNB and 6'-sialylated LNB in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and wherein no precursor needs to be supplied to the
15 cultivation.

Example 26. Production of a mammalian milk oligosaccharide mixture comprising GlcNAc-b1,3-Gal-b1,4-GlcNAc and poly-LacNAc structures with a modified *E. coli* host

An *E. coli* K-12 MG1655 strain is modified with a knock-out of the *E. coli* *nagA* and *nagB* genes together
20 with genomic knock-ins of constitutive transcriptional units for the mutant glmS*54 with SEQ ID NO 19 from *E. coli*, GNA1 with SEQ ID NO 16 from *S. cerevisiae* and LgtB with SEQ ID NO 29 from *N. meningitidis* to produce LacNAc. In a next step, the novel strain is additionally transformed with an expression plasmid containing a constitutive transcriptional unit for the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from *N. meningitidis* with SEQ ID NO 27. By subsequent action of the
25 glmS*54 enzyme and the homologous EcGlmM and EcGlmU enzymes, the mutant strain is capable to produce UDP-GlcNAc, which is used by the heterologous LgtA protein to modify LacNAc into GlcNAc-b1,3-Gal-b1,4-GlcNAc. The novel strain is evaluated for production of said GlcNAc-b1,3-Gal-b1,4-GlcNAc in whole broth samples, in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source. The novel strain is also evaluated for
30 production of poly-LacNAc structures, i.e. (Gal-b1,4-GlcNAc)_n which are built of repeated N-acetylglucosamine units that are beta1,3-linked to each other by alternate activity of the heterologous LgtB and LgtA expressed in the strain.

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Example 27. Production of a mammalian milk oligosaccharide mixture comprising GlcNAc-b1,3-Gal-b1,4-GlcNAc, beta-Gal-(1,4)-beta-GlcNAc-(1,3)-[beta-GlcNAc-(1,6)]-beta-Gal-(1,4)-GlcNAc and poly-LacNAc structures with a modified *E. coli* host

In a next example, the mutant *E. coli* strain adapted to produce LacNAc, UDP-GlcNAc, GlcNAc-b1,3-Gal-b1,4-GlcNAc and (Gal-b1,4-GlcNAc)_n structures as described in Example 26, is further modified with a second expression plasmid containing a constitutive transcriptional unit for the human N-acetylglucosaminide beta-1,6-N-acetylglucosaminyltransferase GCNT2 with SEQ ID NO 32. The novel strain is evaluated for production of an oligosaccharide mixture comprising LacNAc, GlcNAc-b1,3-Gal-b1,4-GlcNAc, GlcNAc-b1,6-Gal-b1,4-GlcNAc, (Gal-b1,4-GlcNAc)_n structures and beta-Gal-(1,4)-beta-GlcNAc-(1,3)-[beta-GlcNAc-(1,6)]-beta-Gal-(1,4)-GlcNAc in whole broth samples, in a growth experiment according to the culture conditions provided in Example 1 in which the culture medium contains glycerol as carbon source.

Example 28. Production of a mammalian milk oligosaccharide mixture comprising Gal-a1,3-LacNAc, LN3, LNnT and Gal-a1,3-LNnT with a modified *E. coli* host

An *E. coli* strain modified to produce LacNAc as described in Example 26 is further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the C-terminal catalytic domain of the bovine alpha-1,3-galactosyltransferase (a3FTcd) with SEQ ID NO 33. The novel strain is evaluated for production of an oligosaccharide mixture comprising Gal-a1,3-LacNAc (Gal-a1,3-Gal-b1,4-GlcNAc), LN3, LNnT and Gal-a1,3-LNnT (Gal-a1,3-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

Example 29. Production of a mammalian milk oligosaccharide mixture comprising LacNAc, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, LN3, LNnT, GalNAc-b1,3-LacNAc, Gal-b1,3-GalNAc-b1,3-LacNAc, poly-LacNAc (Gal-b1,4-GlcNAc)_n and GalNAc-ylated poly-LacNAc structures with a modified *E. coli* host

An *E. coli* strain modified to produce LacNAc as described in Example 26 is further modified with a genomic knock-out of the *E. coli lacZ* gene and with genomic knock-ins of constitutive expression units for the 4-epimerase (WbpP) of *Pseudomonas aeruginosa* with SEQ ID NO 34, the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from *N. meningitidis* with SEQ ID NO 27 and the β1,3-N-acetylglucosaminyltransferase (LgtD) from *Haemophilus influenzae* with SEQ ID NO 35. The novel strain is evaluated for production of an oligosaccharide mixture comprising LacNAc, LN3, LNnT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-LacNAc, Gal-b1,3-GalNAc-b1,3-LacNAc, poly-LacNAc structures, i.e. (Gal-b1,4-GlcNAc)_n which are built of repeated N-acetylglucosamine units that are beta1,3-linked to each other by alternate activity of the N-acetylglucosamine beta-1,4-galactosyltransferase and

the galactoside beta-1,3-N-acetylglucosaminyltransferase, and GalNAc-ylated poly-LacNAc structures in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

5 Example 30. Production of a mammalian milk oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB with a modified E. coli host

An E. coli strain is modified with a genomic knock-out of the E. coli nagB gene and genomic knock-ins of constitutive expression cassettes for the mutant glmS*54 with SEQ ID NO 19 from E. coli, GNA1 with SEQ
10 ID NO 16 from S. cerevisiae and WbgO with SEQ ID NO 28 from E. coli O55:H7 to produce LNB. In a next step, the LNB producing E. coli strain is further modified with a knock-out of the E. coli lacZ gene and with knock-ins of constitutive expression units for the 4-epimerase (WbpP) of P. aeruginosa with SEQ ID NO 34, the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from N. meningitidis with SEQ ID NO 27 and the β 1,3-N-acetylgalactosaminyltransferase (LgtD) from H. influenzae with SEQ ID NO 35. The novel
15 strain is evaluated for production of an oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB, in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium comprises glycerol as carbon source and lactose as precursor.

20 Example 31. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified E. coli host

An E. coli strain adapted for GDP-fucose production as exemplified in Example 1 is further transformed with two compatible expression plasmids wherein a first plasmid contains constitutive expression units for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive
25 expression units for one or two selected sialyltransferase(s) and the N-acylneuraminate cytidyltransferase (NeuA) from P. multocida with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures in whole broth samples as shown in Table 3, in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains
30 sucrose as carbon source and sialic acid and lactose as precursors.

Table 2: Overview of the plasmids with constitutive transcriptional units for one or two fucosyltransferase gene(s) or for one or two sialyltransferase gene(s)

Plasmid	Fucosyltransferase expressed	
	a1,2- linkage	a1,3-linkage
pMF_1A	SEQ ID NO 04	None
pMF_1B	None	SEQ ID NO 05
pMF_2	SEQ ID NO 04	SEQ ID NO 05
Plasmid	Sialyltransferase expressed	
	a2,3-linkage	a2,6-linkage
pMS_1A	SEQ ID NO 23	None
pMS_1B	None	SEQ ID NO 25
pMS_2	SEQ ID NO 23	SEQ ID NO 25

Table 3: Oligosaccharide production evaluated in the mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and sialic acid and lactose as precursors.

Strain	Plasmids* present	Oligosaccharides
SF1	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL
SF2	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL
SF3	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL
SF4	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL
SF5	pMF_1A, pMS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SF6	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SF7	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SF8	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SF9	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 2 for plasmid info

Example 32. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as exemplified in Example 1 is further modified for sialic acid production with genomic knock-outs of the *E. coli* genes *nagA*, *nagB*, *nanA*, *nanE* and *nanK* together with genomic knock-ins of constitutive transcriptional units for the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* of *S. cerevisiae* with SEQ ID NO 16, the N-acetylglucosamine 2-epimerase (AGE) of *Bacteroides ovatus* with SEQ ID NO 17, and the N-acetylneuraminase synthase (*neuB*) of *N. meningitidis*

with SEQ ID NO 18. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures in whole broth samples as shown in Table 4, in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 4: Oligosaccharide production evaluated in the mutant *E. coli* strains in a growth experiment according to the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF10	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL
SF11	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL
SF12	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL
SF13	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL
SF14	pMF_1A, pMS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SF15	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SF16	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SF17	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SF18	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 2 for plasmid info

Example 33. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as exemplified in Example 1 is further modified for sialic acid production with genomic knock-outs of the *E. coli* genes *nagA*, *nagB*, *nanA*, *nanE* and *nanK* together with genomic knock-ins of constitutive transcriptional units for the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, the UDP-N-acetylglucosamine 2-epimerase (*neuC*) of *Campylobacter jejuni* with SEQ ID 21 and the N-acetylneuraminase synthase (*neuB*) of *N. meningitidis* with SEQ ID NO 18. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose

structures in whole broth samples as shown in Table 5, in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

- 5 **Table 5:** Oligosaccharide production evaluated in the mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF19	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL
SF20	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL
SF21	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL
SF22	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL
SF23	pMF_1A, pMS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SF24	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SF25	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SF26	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SF27	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 2 for plasmid info

- 10 Example 34. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as exemplified in Example 1 is further modified via a genomic knock-out of the *E. coli wcaJ* gene to increase the intracellular pool of GDP-fucose. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains

15 (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures in whole broth samples as shown in Table 6, in a growth experiment according to the culture

20 conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 6: Oligosaccharide production evaluated in the mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF28	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL
SF29	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL
SF30	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL
SF31	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL
SF32	pMF_1A, pMS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SF33	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SF34	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SF35	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SF36	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 2 for plasmid info

5

Example 35. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as exemplified in Example 1 is further modified via genomic knock-outs of the *E. coli* *wcaJ*, *fucK* and *fucI* genes and genomic knock-ins of constitutive expression units for the fucose permease (*fucP*) from *E. coli* with SEQ ID NO 13 and the bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase (*fkp*) from *B. fragilis* with SEQ NO ID 14 to increase the intracellular pool of GDP-fucose. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures in whole broth samples as shown in Table 7, in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

20

Table 7: Oligosaccharide production evaluated in the mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF37	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL

SF38	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL
SF39	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL
SF40	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL
SF41	pMF_1A, pMS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SF42	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SF43	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SF44	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SF45	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 2 for plasmid info

Example 36. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNT, 3'SL and LSTa with a modified E. coli host

- 5 An *E. coli* strain modified to produce LNT as described in Example 1 is further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid containing constitutive expression cassettes for NeuA from *P. multocida* with SEQ ID NO 22 and the α -2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23. The novel strain is evaluated for production of a mixture of oligosaccharides comprising LN3, 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNT, 3'SL
10 and LSTa (Neu5Ac-a2,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in a growth experiment according to the culture conditions in a 96-well plate provided in Example 1, in which the culture medium contains glycerol as carbon source and both sialic acid and lactose as precursors. After 72h of incubation, the culture broth is harvested, and the sugar mixtures are analyzed as described in Example 1. .

15 Example 37. Production of a mammalian milk oligosaccharide mixture comprising 6'SL, LN3, sialylated LN3, LNNt and LSTc with a modified E. coli host

- An *E. coli* strain modified to produce LNNt as described in Example 1 was further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid containing constitutive expression cassettes for NeuA from *P. multocida* with SEQ ID NO 22 and one selected α -2,6-sialyltransferase. In this experiment, the α -2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25 and
20 the α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 with SEQ ID NO 26 were tested, wherein the α -2,6-sialyltransferase with SEQ ID NO 26 was expressed from two different transcriptional units. As such, three different strains were created each expressing a single α -2,6-sialyltransferase in a specific transcriptional unit. Table 8 shows an overview of the transcriptional units used for the selected α -2,6-sialyltransferase proteins. The novel strains were evaluated in a growth experiment in a 96-well plate
25 according to the culture conditions provided in Example 1, in which the culture medium contained glycerol as carbon source and both sialic acid and lactose as precursors. After 72h of incubation, the culture broth was harvested, and the sugar mixtures were analyzed as described in Example 1. All novel strains

produced a mixture of oligosaccharides comprising 6'SL, LN3, LNnT and LSTc (Neu5Ac-a2,6-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc). Figure 1 shows the chromatogram obtained for the strain S2 analyzed via the Dionex method as described in Example 1. The compound 6'-sialylated LN3 (Neu5Ac-a2,6-(GlcNAc-b1,3)-Gal-b1,4-Glc) could also be detected in these samples using an UPLC with RI detection as described in Example 1. The ratio of 6'SL, LN3, LNnT and LSTc produced in the mixtures of the new strains could be accurately tuned by the choice of α -2,6-sialyltransferase expressed and the transcriptional units used to express these sialyltransferases.

Table 8: Overview of mutant *E. coli* strains expressing a different α -2,6-sialyltransferase in a specific transcriptional unit.

Strain	Transcriptional unit			
	Promoter	UTR	SEQ ID NO CDS	Terminator
S1	"PROM0005_MutalikP5"	"UTR0010_GalE_BCD12"	25	"TER0007_ilvGEDA"
S2	"PROM0005_MutalikP5"	"UTR0010_GalE_BCD12"	26	"TER0007_ilvGEDA"
S3	"PROM0050_apFAB82"	"UTR0014_GalE_LeuAB"	26	"TER0007_ilvGEDA"

Example 38. Production of a mammalian milk oligosaccharide mixture comprising LN3, LNnT, sialylated LN3, 3'SL and LSTd with a modified *E. coli* host

An *E. coli* strain modified to produce LNnT as described in Example 1 was further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid containing constitutive expression cassettes for NeuA from *P. multocida* with SEQ ID NO 22 and one selected α -2,3-sialyltransferase. In this experiment, the α -2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and the α -2,3-sialyltransferase from *N. meningitidis* with SEQ ID NO 324 were tested, and both α -2,3-sialyltransferases were each cloned in two different transcriptional units. As such, four different strains were created each expressing a single α -2,3-sialyltransferase in a specific transcriptional unit. Table 9 shows an overview of the transcriptional units used for the selected α -2,3-sialyltransferase proteins. The novel strains were evaluated in a growth experiment in a 96-well plate according to the culture conditions provided in Example 1, in which the culture medium contained glycerol as carbon source and both sialic acid and lactose as precursors. After 72h of incubation, the culture broth was harvested, and the sugar mixtures were analyzed as described in Example 1. All novel strains produced a mixture of oligosaccharides comprising 3'SL, LN3, LNnT and LSTd (Neu5Ac-a2,3-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc). Figure 2 shows the chromatogram obtained for the strain S5 analyzed via the Dionex method as described in Example 1. The compound 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc) could also be detected in these samples using an UPLC with RI detection as described in Example 1. The ratio of 3'SL, LN3, LNnT and LSTd produced in the mixtures of the new strains could be accurately tuned by the choice of α -2,3-sialyltransferase expressed and the transcriptional units used to express these sialyltransferases.

Table 9: Overview of mutant *E. coli* strains expressing a different α -2,3-sialyltransferase in a specific transcriptional unit.

Strain	Transcriptional unit			
	Promoter	UTR	SEQ ID NO CDS	Terminator
S4	"PROM0050_apFAB82"	"UTR0014_GalE_LeuAB"	23	"TER0007_ilvGEDA"
S5	"PROM0005_MutalikP5"	"UTR0010_GalE_BCD12"	23	"TER0007_ilvGEDA"
S6	"PROM0050_apFAB82"	"UTR0014_GalE_LeuAB"	24	"TER0007_ilvGEDA"
S7	"PROM0005_MutalikP5"	"UTR0010_GalE_BCD12"	24	"TER0007_ilvGEDA"

Example 39. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNT, 3'SL and LSTa with a modified *E. coli* host

An *E. coli* strain modified to produce sialic acid as described in Example 1 is further modified with a genomic knock-in of constitutive transcriptional units for the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from *N. meningitidis* with SEQ ID NO 27 and for the N-acetylglucosamine beta-1,3-galactosyltransferase (WbgO) from *E. coli* O55:H7 with SEQ ID NO 28 to allow production of LNT. In a next step, the novel strain is further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid having constitutive transcriptional units for NeuA from *P. multocida* with SEQ ID NO 22 and the α -2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNT, 3'SL and LSTa in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 40. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNnT, 6'SL and LSTc with a modified *E. coli* host

An *E. coli* strain modified to produce sialic acid as described in Example 1 was further modified with a genomic knock-in of constitutive transcriptional units for LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29 to allow production of LNnT. In a next step, the novel strain was further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid having constitutive transcriptional units for NeuA from *P. multocida* with SEQ ID NO 22 and the α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 with SEQ ID NO 26. The novel strain was evaluated in a growth experiment in a 96-well plate according to the culture conditions provided in Example 1, in which the culture medium contained sucrose as carbon source and lactose as precursor. After 72h of incubation, the culture broth was harvested, and the sugar mixtures were analyzed on UPLC. The experiment showed that the novel strain produced a mixture of oligosaccharides comprising LN3, 6'-sialylated LN3 (Neu5Ac-a2,6-(GlcNAc-b1,3)-Gal-b1,4-Glc), 6'SL, LNnT and LSTc.

Example 41. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNnT, 3'SL and LSTd with a modified E. coli host

An *E. coli* strain modified to produce sialic acid as described in Example 1 is further modified with a genomic knock-in of constitutive transcriptional units for LgtA from *N. meningitidis* with SEQ ID NO 27 and for LgtB from *N. meningitidis* with SEQ ID NO 29 to allow production of LNnT. In a next step, the novel strain is further modified with a genomic knock-out of the *E. coli lacZ* gene and transformed with an expression plasmid having constitutive transcriptional units for NeuA from *P. multocida* with SEQ ID NO 22 and the α -2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNnT, 3'SL and LSTd in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 42. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNT, LSTa and 3'SL in fermentation broth of mutant E. coli strains when evaluated in a fed-batch fermentation process with glycerol, sialic acid and lactose

The mutant *E. coli* strain able to produce LN3, LNT, 3'SL and LSTa as described in Example 36 is selected for further evaluation in a fed-batch fermentation process in a 5L bioreactor. Fed-batch fermentations at bioreactor scale are performed as described in Example 1. In these examples, glycerol is used as a carbon source and lactose is added in the batch medium as precursor. During fed-batch, also sialic acid is added via an additional feed. In contrast to the cultivation experiments that are described herein and wherein only end samples were taken at the end of cultivation (i.e. 72 hours as described herein), regular broth samples are taken at several time points during the fermentation process and evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNT, LSTa and 3'SL.

Example 43. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNT, LSTa and 3'SL in fermentation broth of mutant E. coli strains when evaluated in a fed-batch fermentation process with sucrose and lactose

The mutant *E. coli* strain able to produce LN3, LNT, 3'SL and LSTa as described in Example 39 is selected for further evaluation in a fed-batch fermentation process in a 5L bioreactor. Fed-batch fermentations at bioreactor scale are performed as described in Example 1. In these examples, sucrose is used as a carbon source and lactose is added in the batch medium as precursor. In contrast to the cultivation experiments that are described herein and wherein only end samples were taken at the end of cultivation (i.e. 72 hours as described herein), regular broth samples are taken at several time points during the fermentation process and evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3 (Neu5Ac-a2,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNT, LSTa and 3'SL.

Example 44. Production of a mammalian milk oligosaccharide mixture comprising LN3, sialylated LN3, LNnT, para-lacto-N-neohexaose, di-sialylated LNnT, LSTc and 6'SL in fermentation broth of mutant E. coli strains when evaluated in a fed-batch fermentation process with glycerol, sialic acid and lactose

The mutant *E. coli* strains with a constitutive transcriptional unit for the α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 with SEQ ID NO 26 as described in Example 37 were selected for further evaluation in a fed-batch fermentation process in a 5L bioreactor. Fed-batch fermentations at bioreactor scale were performed as described in Example 1. In these examples, glycerol is used as a carbon source and lactose was added in the batch medium as precursor. During fed-batch, also sialic acid was added via an additional feed. Regular broth samples were taken, and sugars produced were measured as described in Example 1. UPLC analysis shows that fermentation broth of the selected strain taken after the batch phase contains lactose, LN3 and LNnT, whereas fermentation broth of the selected strain taken after the fed-batch phase comprises an oligosaccharide mixture comprising LN3, 6'-sialylated LN3 (Neu5Ac-a2,6-(GlcNAc-b1,3)-Gal-b1,4-Glc), LNnT, LSTc and 6'SL. At end of fed-batch, the mixture also comprises para-lacto-N-neohexaose (pLNnH), sialylated para-lacto-N-neohexaose, and di-sialylated LNnT, i.e. structures that were not detected in growth experiment assays due to limited detection levels and overall smaller production levels.

Example 45. Production of an oligosaccharide mixture comprising LN3, sialylated LN3, LNnT, para-lacto-N-neohexaose, di-sialylated LNnT, LSTc and 6'SL in fermentation broth of mutant E. coli strains when evaluated in a fed-batch fermentation process with sucrose and lactose

The mutant *E. coli* strain able to produce LN3, sialylated LN3, LNnT, 6'SL and LSTc as described in Example 40 was selected for further evaluation in a fed-batch fermentation process in a 5L bioreactor. Fed-batch fermentations at bioreactor scale were performed as described in Example 1. In these examples, sucrose was used as a carbon source and lactose was added in the batch medium as precursor. Regular broth samples were taken, and sugars produced were measured as described in Example 1. UPLC analysis shows that fermentation broth of the selected strain taken after the batch phase contains lactose, LN3, 6'SL, and LNnT, whereas fermentation broth of the selected strain taken after the fed-batch phase comprises an oligosaccharide mixture comprising LN3, 6'-sialylated LN3 (Neu5Ac-a-2,6-(GlcNAc-b-1,3)-Gal-b-1,4-Glc), LNnT, LSTc and 6'SL. At end of fed-batch, the mixture also comprises para-lacto-N-neohexaose, sialylated para-lacto-N-neohexaose and di-sialylated LNnT, two structures that were not detected in growth experiment assays due to limited detection levels and overall smaller production levels.

Example 46. Production of a mammalian milk oligosaccharide mixture comprising LNT, LNnT and poly-galactosylated structures in a modified E. coli host when evaluated in fed-batch fermentations

An *E. coli* strain modified to produce LNnT as described in Example 1, is further modified with genomic knock-ins of constitutive transcriptional units for WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next

step, the novel strain is evaluated in a fed-batch fermentation process in a 5L bioreactor as described in Example 1. In this example sucrose is used as a carbon source and lactose is added in the batch medium as precursor. In contrast to the cultivation experiments that are described herein and wherein only end samples were taken at the end of cultivation (i.e. 72 hours as described herein), regular broth samples are taken at several time points during the fermentation process and evaluated for production of an oligosaccharide mixture comprising Lacto-N-triose II (LN3), Lacto-N-neotetraose (LNnT), Lacto-N-tetraose (LNT), para-Lacto-N-neopentaose, para-Lacto-N-pentaose, para-Lacto-N-neohexaose, para-Lacto-N-hexaose, beta-(1,3)Galactosyl-para-Lacto-N-neopentaose and beta-(1,4)Galactosyl-para-Lacto-N-pentaose.

Example 47. Production of a mammalian milk oligosaccharide mixture comprising galactosylated and GalNAc-ylated lactose structures with a modified *E. coli* host

An *E. coli* strain optimized for UDP-galactose as described in Example 1, is further modified with a knock-out of the *E. coli lacZ* gene and a knock-in of a constitutive expression unit for the α 1,4-galactosyltransferase (LgtC) from *Neisseria gonorrhoeae* with SEQ ID NO 36 to produce α 1,4-galactosylated lactose (Gal- α 1,4-Gal-b1,4-Glc) when grown on glycerol and lactose. In a next step, the mutant strain is further modified with a knock-out of the *E. coli nagB* gene together with a knock-in of a constitutive expression unit for the mutant glmS*54 of *E. coli* with SEQ ID NO 19, and transformed with an expression plasmid comprising constitutive expression units for the 4-epimerase (WbpP) of *P. aeruginosa* with SEQ ID NO 34 and the β 1,3-N-acetylgalactosaminyltransferase (LgtD) from *H. influenzae* with SEQ ID NO 35. Besides its β 1,3-N-Acetyl-galactosaminyltransferase activity the LgtD enzyme also has a β 1,3-galactosyltransferase activity and can add a galactose to a non-reducing terminal GalNAc molecule resulting in a terminal Gal-b1,3-GalNAc at the non-reducing end of a glycan. The novel strain is evaluated for production of an oligosaccharide mixture comprising Gal- α 1,4-Gal-b1,4-Glc (Gal- α 1,4-lactose), Gal- α 1,4-Gal- α 1,4-Gal-b1,4-Glc, Gal- α 1,4-Gal- α 1,4-Gal- α 1,4-Gal-b1,4-Glc, GalNAc-b1,3-Gal-b1,4-Glc (GalNAc-b1,3-Lactose), Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-Gal- α 1,4-Gal-b1,4-Glc (globo-N-tetraose) and Gal-b1,3-GalNAc-b1,3-Gal- α 1,4-Gal-b1,4-Glc in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

Example 48. Production of an oligosaccharide mixture comprising LN3, LNT, GalNAc-b1,3-LNT, Gal-b1,3-GalNAc-b1,3-LNT, GalNAc-b1,3-lactose and Gal-b1,3-GalNAc-b1,3-lactose with a modified *E. coli* host

An *E. coli* strain modified to produce LNT as described in Example 1, is further modified with knock-ins of constitutive expression units for the 4-epimerase (WbpP) from *P. aeruginosa* with SEQ ID NO 34 and the β 1,3-N-acetylgalactosaminyltransferase (LgtD) from *H. influenzae* with SEQ ID NO 35. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, LNT, GalNAc-b1,3-LNT, Gal-b1,3-

GalNAc-b1,3-LNT, GalNAc-b1,3-lactose and Gal-b1,3-GalNAc-b1,3-lactose in whole broth samples in a growth experiment, according to the culture conditions in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

5 Example 49. Production an oligosaccharide mixture comprising LN3, LNnT, GalNAc-b1,3-LNnT, Gal-b1,3-GalNAc-b1,3-LNnT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose and (GalNAc-)poly-LNnT structures with a modified E. coli host

An E. coli strain modified to produce LNnT as described in Example 1, is further modified with knock-ins of constitutive expression units for WbpP from P. aeruginosa with SEQ ID NO 34 and LgtD from H. influenzae with SEQ ID NO 35. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, LNnT, GalNAc-b1,3-LNnT, Gal-b1,3-GalNAc-b1,3-LNnT, GalNAc-b1,3-lactose and Gal-b1,3-GalNAc-b1,3-lactose as well as poly-LNnT structures (by alternate activity of NmlgtA and NmLgtB) and GalNAc-ylated poly-LNnT structures (by additional activity of HiLgtD) in whole broth samples in a growth experiment, according to the culture conditions in Example 1, in which the culture medium contains glycerol as carbon source and lactose as precursor.

Example 50. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and LNFP-I with a modified E. coli host

An E. coli strain modified for GDP-fucose production as described in Example 1 was further adapted for LN3 and LNT production by genomic knock-ins of constitutive transcriptional units for the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from N. meningitidis with SEQ ID NO 27 and the N-acetylglucosamine beta-1,3-galactosyltransferase (WbgO) from E. coli O55:H7 with SEQ ID NO 28. In a next step, the novel strain was further transformed with an expression plasmid containing a constitutive transcriptional unit for the a-1,2-fucosyltransferase from H. pylori with SEQ ID NO 04 that accepts both LNT and lactose as acceptors for its fucosyltransferase activity. The novel strain produced an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and lacto-N-fucopentaose I (LNFP-I, Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples when evaluated in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contained sucrose as carbon source and lactose as precursor.

Example 51. Production of an oligosaccharide mixture comprising LN3, LNT and LNFP-I with a modified E. coli host

An E. coli K-12 MG1655 strain modified for GDP-fucose production as described in Example 1 is further adapted for LN3 and LNT production by genomic knock-outs of the E. coli nagB and arabinose isomerase (araA) genes and genomic knock-ins of constitutive transcriptional units for galE from E. coli with SEQ ID NO 30, the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from N. meningitidis with SEQ ID

NO 27 and the N-acetylglucosamine beta-1,3-galactosyltransferase (WbdO) from *Salmonella enterica* subsp. *salamae* serovar Greenside with SEQ ID NO 41. In a next step, the novel strain is further transformed with an expression plasmid containing a constitutive transcriptional unit for the α -1,2-fucosyltransferase (fucT54) from *Sideroxydans lithotrophicus* ES-1 with SEQ ID NO 40. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, LNT and lacto-N-fucopentaose I (LNFP-I, Fuc- α 1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

10 Example 52. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, GalNAc-b1,3-lactose, LN3, LNT, GalNAc-b1,3-LNT, LNFP-I and GalNAc-b1,3-LNFP-I with a modified *E. coli* host

An *E. coli* strain modified for GDP-fucose production and for LNFP-I production as described in Example 50 is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the b1,3-N-acetylgalactosaminyltransferase (LgtD) from *H. influenzae* with SEQ ID NO 35. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, GalNAc-b1,3-Lactose (GalNAc-b1,3-Gal-b1,4-Glc), LN3, LNT, GalNAc-b1,3-LNT (GalNAc-b1,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc), LNFP-I (Fuc- α 1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) and GalNAc-b1,3-LNFP-I (GalNAc-b1,3-(Fuc- α 1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

20 Example 53. Production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I and Gal-a1,3-(Fuc- α 1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc with a modified *E. coli* host

An *E. coli* strain modified for GDP-fucose production and for LNFP-I production as described in Example 50 is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the α -1,3-galactosyltransferase Wbni from *E. coli* with SEQ ID NO 37. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I (Fuc- α 1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) and Gal-a1,3-LNFP-I (Gal-a1,3-(Fuc- α 1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

30 Example 54. Production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I and GalNAc-a1,3-(Fuc- α 1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc with a modified *E. coli* host

An *E. coli* strain modified for GDP-fucose production and for LNFP-I production as described in Example 50 is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the α -1,3-N-acetylgalactosaminyltransferase BgtA from *H. mustelae* with SEQ ID NO 38. The

novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I (Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) and GalNAc-a1,3-LNFP-I (GalNAc-a1,3-(Fuc-a1,2)-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 55. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT and LNFP-I with a modified E. coli host

An *E. coli* strain modified for GDP-fucose and LNFP-I production comprising expression of glmS*54 from *E. coli* with SEQ ID NO 19, LgtA from *N. meningitidis* with SEQ ID NO 27, WbgO from *E. coli* O55:H7 with SEQ ID NO 28 and the a-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 as described in Example 50, is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the a-1,3-fucosyltransferase (HpFucT) from *H. pylori* with SEQ ID NO 05. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT and LNFP-I (Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 56. Production of a mammalian milk oligosaccharide mixture comprising LN3, LNT and LNFP-II with a modified E. coli host

An *E. coli* strain modified for GDP-fucose production as described in Example 1 is further adapted for LN3 and LNT production by a genomic knock-out of the *E. coli nagB* gene and genomic knock-ins of constitutive transcriptional units for LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is further transformed with an expression plasmid containing a constitutive transcriptional unit for a mutant a1,3/4 fucosidase from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 with SEQ ID NO 39. The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, LNT and lacto-N-fucopentaose II (LNFP-II, Gal-b1,3-(Fuc-a1,4)-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 57. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I and LNFP-II with a modified E. coli host

An *E. coli* strain modified for GDP-fucose production and for LNFP-II production as described in Example 56 is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the a-1,2-fucosyltransferase (HpFutC) from *H. pylori* with SEQ ID NO 04. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT, LNFP-I and lacto-

N-fucopentaose II (LNFP-II, Gal-b1,3-(Fuc-a1,4)-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

5 Example 58. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LN3, LNT and LNFP-II with a modified E. coli host

An *E. coli* strain modified for GDP-fucose production and for LNFP-II production as described in Example 56 is further transformed with a compatible expression plasmid containing a constitutive transcriptional unit for the a-1,3-fucosyltransferase (HpFucT) from *H. pylori* with SEQ ID NO 05. The novel strain is
10 evaluated for production of an oligosaccharide mixture comprising 3-FL, LN3, LNT and lacto-N-fucopentaose II (LNFP-II, Gal-b1,3-(Fuc-a1,4)-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

15 Example 59. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT, LNFP-I and LNFP-II with a modified E. coli host

An *E. coli* strain modified for GDP-fucose production and for LNFP-II production as described in Example 56 is further transformed with a compatible expression plasmid containing constitutive transcriptional units for the a-1,2-fucosyltransferase (HpFutC) from *H. pylori* with SEQ ID NO 04 and for the a-1,3-fucosyltransferase (HpFucT) from *H. pylori* with SEQ ID NO 05. The novel strain is evaluated for production
20 of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT, LNFP-I and lacto-N-fucopentaose II (LNFP-II, Gal-b1,3-(Fuc-a1,4)-GlcNAc-b1,3-Gal-b1,4-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

25 Example 60. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LN3, LNT and LNFP-V with a modified E. coli host

An *E. coli* strain modified for GDP-fucose production as described in Example 1 is further adapted for LN3 and LNT production by a genomic knock-out of the *E. coli nagB* gene and genomic knock-ins of constitutive
30 transcriptional units for LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is further transformed with an expression plasmid containing a constitutive transcriptional unit for the truncated a1,3-fucosyltransferase from *H. pylori* with SEQ ID NO 06. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3-FL, LN3, LNT and lacto-N-fucopentaose V (LNFP-V, Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-(Fuca1,3)-Glc) in whole broth
35 samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 61. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LN3, LNnT, LNFP-III and LNFP-VI with a modified *E. coli* host

An *E. coli* strain modified for GDP-fucose production as described in Example 1 is further adapted for LN3 and LNnT production by a genomic knock-out of the *E. coli nagB* gene and genomic knock-ins of constitutive transcriptional units for LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is further transformed with an expression plasmid containing a constitutive transcriptional unit for the truncated α -1,3-fucosyltransferase from *H. pylori* with SEQ ID NO 06. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3-FL, LN3 and LNnT, lacto-N-fucopentaose III (LNFP-III, Gal-b1,4-(Fuc-a1,3)-GlcNAc-b1,3-Gal-b1,4-Glc) and lacto-N-fucopentaose VI (LNFP-VI, Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-(Fuc-a1,3)-Glc) in whole broth samples in a growth experiment according to the culture conditions provided in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Example 62. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with a genomic knock-out of the *E. coli nagB* gene and genomic knock-ins of constitutive expression cassettes for LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 10), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and sialic acid and lactose as precursors.

Table 10: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and sialic acid and lactose as precursors.

Strain	Plasmids* present	Oligosaccharides
SF46	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF47	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, LNT, 3'S-LN3, LSTa
SF48	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNT, LNFP-I
SF49	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNT

SF50	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF51	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF52	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF53	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNT, LNFP-I
SF54	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

Example 63. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

- 5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with a genomic knock-out of the *E. coli* *nagB* gene and genomic knock-ins of constitutive expression cassettes for LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a
- 10 second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 11), in a growth experiment according to the culture conditions provided in Example 1 in
- 15 which the cultivation contains sucrose as carbon source and sialic acid and lactose as precursors.

Table 11: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in

20 Example 1, in which the culture medium contains sucrose as carbon source and sialic acid and lactose as precursors.

Strain	Plasmids* present	Oligosaccharides
SF55	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNnT, LSTd
SF56	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF57	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNnT, LSTc
SF58	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF59	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd

SF60	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF61	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF62	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF63	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 64. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE* and *nank* genes and genomic knock-ins of constitutive expression cassettes for the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* from *S. cerevisiae* with SEQ ID NO 16, *AGE* of *B. ovatus* with SEQ ID NO 17, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In

10 a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising

15 fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 12), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

20 **Table 12:** Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF64	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF65	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF66	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF67	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF68	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB,

		6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF69	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF70	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF71	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF72	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

Example 65. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

- 5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE* and *nank* genes and genomic knock-ins of constitutive expression cassettes for the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* from *S. cerevisiae* with SEQ ID NO 16, *AGE* of *B. ovatus* with SEQ ID NO 17, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 29. In
- 10 a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising
- 15 fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNNt and fucosylated and sialylated LNNt structures in whole broth samples (Table 13), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

- 20 **Table 13:** Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF73	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNNt, LSTd

SF74	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FlacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF75	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF76	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FlacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF77	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF78	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FlacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF79	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF80	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF81	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 66. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

- 5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE* and *nanK* genes and genomic knock-ins of constitutive expression cassettes for the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, the UDP-N-acetylglucosamine 2-epimerase (*neuC*) from *C. jejuni* with SEQ ID 21, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In
- 10 a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising
- 15 fucosylated and sialylated lactose, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples as shown in Table 14, in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 14: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF82	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF83	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LSTa
SF84	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNT, LNFP-I
SF85	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNT
SF86	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF87	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF88	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF89	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNT, LNFP-I
SF90	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, LNT, 3'S-LN3, 6'S-LN3, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 67. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE* and *nanK* genes and genomic knock-ins of constitutive expression cassettes for the mutant *glms**54 from *E. coli* with SEQ ID NO 19, *neuC* from *C. jejuni* with SEQ ID 21, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 15), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 15: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF91	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNnT, LSTd
SF92	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF93	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNnT, LSTc
SF94	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF95	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF96	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF97	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF98	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF99	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

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Example 68. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with a genomic knock-out of the *E. coli wcaJ* gene to increase the intracellular pool of GDP-fucose and genomic knock-ins of constitutive expression cassettes for LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 16), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 16: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according to the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF100	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF101	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF102	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, LNT, 6'S-LN3, LNFP-I
SF103	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF104	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF105	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF106	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, LNT, 3'S-LN3, LNFP-I, LSTa
SF107	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF108	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 69. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with a genomic knock-out of the *E. coli wcaJ* gene to increase the intracellular pool of GDP-fucose and genomic knock-ins of constitutive expression cassettes for LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 17), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 17: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF109	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LSTd
SF110	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FlacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF111	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF112	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FlacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF113	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF114	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FlacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF115	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF116	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF117	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

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Example 70. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli wcaJ*, *fucK* and *fucI* genes and genomic knock-ins of constitutive expression cassettes for the fucose permease (*fucP*) from *E. coli* with SEQ ID NO 13, the bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase (*fkp*) from *B. fragilis* with SEQ NO ID 14, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are

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evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 18), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 18: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF118	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF119	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF120	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF121	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF122	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF123	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF124	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF125	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF126	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, LNT, 3'S-LN3, 6'S-LN3, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 71. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *wcaJ*, *fucK* and *fucI* genes and genomic knock-ins of constitutive expression cassettes for fucP from *E. coli* with SEQ ID NO 13, fkp from *B. fragilis* with SEQ NO ID 14, LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from

15

P. multocida with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 19), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 19: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according to the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF127	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LSTd
SF128	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF129	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF130	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF131	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF132	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF133	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF134	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF135	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 72. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID

NO 19, GNA1 from *S. cerevisiae* with SEQ ID NO 16, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 20), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and sialic acid and lactose as precursors.

Table 20: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and sialic acid and lactose as precursors.

Strain	Plasmids* present	Oligosaccharides
SF136	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF137	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF138	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF139	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF140	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF141	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF142	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF143	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF144	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, LNT, 3'S-LN3, 6'S-LN3, LNFP-I, LSTa

*See Table 2 for plasmid info

Example 73. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* from *S. cerevisiae* with SEQ ID NO 16, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 21), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and sialic acid and lactose as precursors.

Table 21: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and sialic acid and lactose as precursors.

Strain	Plasmids* present	Oligosaccharides
SF145	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'SLN, LNnT, LSTd
SF146	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FlacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF147	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF148	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FlacNAc, 6'SlacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF149	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF150	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FlacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF151	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FlacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd

SF152	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF153	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 74. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE*, *nanK*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* from *S. cerevisiae* with SEQ ID NO 16, the N-acetylglucosamine 2-epimerase (AGE) of *B. ovatus* with SEQ ID NO 17, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N.*

10 *meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are

15 evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 22), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

20 **Table 22:** Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF154	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF155	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF156	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF157	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF158	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

SF159	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF160	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF161	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF162	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

Example 75. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE*, *nanK*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *GNA1* from *S. cerevisiae* with SEQ ID NO 16, *AGE* of *B. ovatus* with SEQ ID NO 17, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from

10 *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide

15 mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 23), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

20 **Table 23:** Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF163	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LSTd
SF164	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT,

		LNFP-III, LSTd
SF165	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF166	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF167	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF168	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF169	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF170	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF171	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 76. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

- 5 An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE*, *nank*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *neuC* from *C. jejuni* with SEQ ID 21, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In a next
- 10 step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and
- 15 sialylated lactose, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 24), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 24: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according to the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF172	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF173	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LSTa
SF174	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNT, LNFP-I
SF175	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNT
SF176	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF177	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF178	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF179	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNT, LNFP-I
SF180	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 77. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for GDP-fucose production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *nagA*, *nagB*, *nanA*, *nanE*, *nanK*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, the mutant *glmS**54 from *E. coli* with SEQ ID NO 19, *neuC* from *C. jejuni* with SEQ ID 21, *neuB* of *N. meningitidis* with SEQ ID NO 18, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 25), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 25: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharide mixture comprising
SF181	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LN3, 3'S-LN3, LNnT, LSTd
SF182	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF183	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LN3, 6'S-LN3, LNnT, LSTc
SF184	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF185	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF186	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF187	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF188	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF189	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

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Example 78. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli wcaJ*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 26), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 26: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF190	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF191	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF192	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF193	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF194	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF195	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF196	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF197	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'SLN, LNT, LNFP-I
SF198	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 79. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli wcaJ*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 29. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 27), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 27: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF199	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LSTd
SF200	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FlacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF201	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF202	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FlacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF203	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF204	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FlacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF205	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF206	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF207	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

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Example 80. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli wcaJ*, *fucK*, *fucI*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, *fucP* from *E. coli* with SEQ ID NO 13, *fkp* from *B. fragilis* with SEQ NO ID 14, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *WbgO* from *E. coli* O55:H7 with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for one or two selected sialyltransferase(s) and *NeuA* from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide

mixture comprising fucosylated and sialylated lactose, LNB, fucosylated and sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures in whole broth samples (Table 28), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

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Table 28: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF208	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LNB, 2'FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF209	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LNB, 4-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LSTa
SF210	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LNB, 2'FLNB, 6'SLNB, LN3, 6'S-LN3, LNT, LNFP-I
SF211	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LNB, 4-FLNB, 6'SLNB, LN3, 6'S-LN3, LNT
SF212	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LNB, 2'FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa
SF213	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LNB, 4-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa
SF214	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, LN3, 3'S-LN3, LNT, LNFP-I, LSTa
SF215	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 6'SLNB, LN3, 6'SLN, LNT, LNFP-I
SF216	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LNB, 2'FLNB, 4-FLNB, Di-FLNB, 3'SLNB, 6'SLNB, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa

*See Table 2 for plasmid info

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Example 81. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *E. coli* host

An *E. coli* strain adapted for sialic acid production as described in Example 1 is further modified with genomic knock-outs of the *E. coli* *wcaJ*, *fucK*, *fucI*, *ushA* and *galT* genes and genomic knock-ins of constitutive expression cassettes for *galE* from *E. coli* with SEQ ID NO 30, *fucP* from *E. coli* with SEQ ID NO 13, *fkp* from *B. fragilis* with SEQ NO ID 14, *LgtA* from *N. meningitidis* with SEQ ID NO 27 and *LgtB* from *N. meningitidis* with SEQ ID NO 28. In a next step, the novel strain is transformed with two compatible expression plasmids wherein a first plasmid contains (a) constitutive expression unit(s) for one or two selected fucosyltransferase(s) and wherein a second plasmid contains constitutive expression units for

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one or two selected sialyltransferase(s) and NeuA from *P. multocida* with SEQ ID NO 22. Table 2 presents an overview of the six plasmids used. The novel strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, fucosylated and sialylated LacNAc, LN3, sialylated LN3, LNnT and fucosylated and sialylated LNnT structures in whole broth samples (Table 29), in a growth experiment according to the culture conditions provided in Example 1 in which the cultivation contains sucrose as carbon source and lactose as precursor.

Table 29: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *E. coli* strains in a growth experiment according the cultivation conditions as described in Example 1, in which the culture medium contains sucrose as carbon source and lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SF217	pMF_1A, pMS_1A	2'FL, 3'SL, 3'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LSTd
SF218	pMF_1B, pMS_1A	3-FL, 3'SL, 3'S-3-FL, LacNAc, 3'FLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF219	pMF_1A, pMS_1B	2'FL, 6'SL, 6'S-2'FL, DiFL, LacNAc, 2'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LSTc
SF220	pMF_1B, pMS_1B	3-FL, 6'SL, 6'S-3-FL, LacNAc, 3'FLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF221	pMF_1A, pMS_2	2'FL, 3'SL, DiFL, 3'S-2'FL, 6'SL, 6'S-2'FL, di-SL, LacNAc, 2'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd
SF222	pMF_1B, pMS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL, di-SL, LacNAc, 3'FLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd
SF223	pMF_2, pMS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd
SF224	pMF_2, pMS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 6'SLacNAc, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc
SF225	pMF_2, pMS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, di-SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LacNAc, 2'FLacNAc, 3'FLacNAc, DiFLacNAc, 3'SLacNAc, 6'SLacNAc, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd

*See Table 2 for plasmid info

Example 82. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for GDP-fucose production and fucosyltransferase expression as described in Example 2 with a yeast expression plasmid (a variant of p2a_2μ_Fuc) comprising constitutive

transcriptional units for the lactose permease (LAC12) from *K. lactis* with SEQ ID NO 31, the GDP-mannose 4,6-dehydratase (gmd) from *E. coli* with SEQ ID NO 11, the GDP-L-fucose synthase (fcl) from *E. coli* with SEQ ID NO 12, the alpha-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and the alpha-1,3-fucosyltransferase from *H. pylori* with SEQ ID NO 05. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL and DiFL in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura drop-out medium comprising lactose as precursor.

Example 83. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated lactose structures with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose and CMP-sialic acid and for expression of one or more fucosyltransferases and one or more sialyltransferases as described in Example 2 with a first yeast expression plasmid (a variant of p2a_2μ_Fuc) comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and one or two selected fucosyltransferase(s) and with a second yeast expression plasmid (a pRS420-plasmid variant) comprising constitutive transcriptional units for the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, neuB from *N. meningitidis* with SEQ ID NO 18, neuA from *P. multocida* with SEQ ID NO 22 and one or two selected sialyltransferase(s). Table 30 shows the fucosyltransferases and sialyltransferases selected in the plasmids cloned in this experiment. The mutant yeast strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures, as shown in Table 31, in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-Trp drop-out medium comprising lactose as precursor.

Table 30: Overview of the plasmids cloned with constitutive transcriptional units for one or two fucosyltransferase gene(s) or for one or two sialyltransferase gene(s)

Plasmid nr	Fucosyltransferase(s) cloned in the p2a_2μ_Fuc plasmid variants	
	a1,2- linkage	a1,3-linkage
pYF_1A	SEQ ID NO 04	None
pYF_1B	None	SEQ ID NO 05
pYF_2	SEQ ID NO 04	SEQ ID NO 05
Plasmid nr	Sialyltransferase(s) cloned in the pRS420-plasmid variant	
	a2,3-linkage	a2,6-linkage
pYS_1A	SEQ ID NO 23	None
pYS_1B	None	SEQ ID NO 25
pYS_2	SEQ ID NO 23	SEQ ID NO 25

Table 31: Oligosaccharide production evaluated by mutant *S. cerevisiae* strains expressing selected fucosyltransferase and sialyltransferase genes and cultivated in SD CSM-Ura-Trp drop-out medium comprising lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SY1	p YF_1A, p YS_1A	2'FL, 3'SL, 3'S-2'FL
SY2	p YF_1B, p YS_1A	3-FL, 3'SL, 3'S-3-FL
SY3	p YF_1A, p YS_1B	2'FL, 6'SL, 6'S-2'FL
SY4	p YF_1B, p YS_1B	3-FL, 6'SL, 6'S-3-FL
SY5	p YF_1A, p YS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SY6	p YF_1B, p YS_2	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SY7	p YF_2, p YS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SY8	p YF_2, p YS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SY9	p YF_2, p YS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 30 for plasmid info

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Example 84. Production of a mammalian milk oligosaccharide mixture comprising LN3, 3'-sialylated LN3, LNT, LNB, 3'SL and LSTa with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of CMP-sialic acid and LNT and for expression of a beta-galactoside alpha-2,3-sialyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, NeuB from *N. meningitidis* with SEQ ID NO 18, NeuA from *P. multocida* with SEQ ID NO 22, and the beta-galactoside alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3, LNT, LNB, 3'SL and LSTa in a growth experiment according to the culture conditions in Example 2 using SD CSM-Trp-His drop-out medium comprising lactose as precursor.

Example 85. Production of a mammalian milk oligosaccharide mixture comprising LN3, 6'-sialylated LN3, LNnT, LacNAc, 6'SL and LSTc with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of CMP-sialic acid and LNnT and for expression of a beta-galactoside alpha-2,6-sialyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, NeuB from *N. meningitidis* with SEQ ID NO 18, NeuA from *P. multocida* with

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SEQ ID NO 22, and the beta-galactoside alpha-2,6-sialyltransferase from *P. damsela* with SEQ ID NO 25 and a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising LN3, 6'-sialylated LN3, LNnT, LacNAc, 6'SL and LSTc in a growth experiment according to the culture conditions in Example 2 using SD CSM-Trp-His drop-out medium comprising lactose as precursor.

Example 86. Production of a mammalian milk oligosaccharide mixture comprising LN3, 3'-sialylated LN3, LNnT, LacNAc, 3'SL and LSTd with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of CMP-sialic acid and LNnT and for expression of a beta-galactoside alpha-2,3-sialyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, NeuB from *N. meningitidis* with SEQ ID NO 18, NeuA from *P. multocida* with SEQ ID NO 22, and the beta-galactoside alpha-2,3-sialyltransferase from *P. multocida* with SEQ ID NO 23 and a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising LN3, 3'-sialylated LN3, LNnT, LacNAc, 3'SL and LSTd in a growth experiment according to the culture conditions in Example 2 using SD CSM-Trp-His drop-out medium comprising lactose as precursor.

Example 87. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and LNFP-I with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose and LNT and for expression of an a-1,2-fucosyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and the a-1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and with a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and lacto-N-fucopentaose I (LNFP-I, Fuc-a1,2-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-His drop-out medium comprising lactose as precursor.

Example 88. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and LNFP-II with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose and LNT and for expression of an α 1,3/4-fucosidase and an α -1,2-fucosyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12, a mutant α 1,3/4-fucosidase from *B. longum subsp. infantis* ATCC 15697 with SEQ ID NO 39 and the α -1,2-fucosyltransferase from *H. pylori* with SEQ ID NO 04 and with a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and lacto-N-fucopentaose II (LNFP-II, Gal-b1,3-(Fuc- α 1,4)-GlcNAc-b1,3-Gal-b1,4-Glc) in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-His drop-out medium comprising lactose as precursor.

Example 89. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LN3, LNT and LNFP-V with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose and LNT and for expression of an α -1,3-fucosyltransferase as described in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and the truncated α 1,3-fucosyltransferase from *H. pylori* with SEQ ID NO 06 and with a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising 3-FL, LN3, LNT and lacto-N-fucopentaose V (LNFP-V, Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-(Fuc α 1,3)-Glc) in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-His drop-out medium comprising lactose as precursor.

Example 90. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LN3, LNnT and LNFP-III with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose and LNnT and for expression of an α -1,3-fucosyltransferase in Example 2 with a first yeast expression plasmid comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and the truncated α 1,3-fucosyltransferase from *H. pylori* with SEQ ID NO 06 and with a second yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. The mutant yeast strain is evaluated for production of an oligosaccharide mixture

comprising 3-FL, LN3, LNnT and lacto-N-fucopentaose III (LNFP-III, Gal-b1,4-(Fuc-a1,3)-GlcNAc-b1,3-Gal-b1,4-Glc) in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-His drop-out medium comprising lactose as precursor.

5 Example 91. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *S. cerevisiae* host

An *S. cerevisiae* strain is adapted for production of GDP-fucose, CMP-sialic acid and LNT and for expression of selected fucosyltransferases and sialyltransferases as described in Example 2 with a first yeast expression plasmid (a variant of p2a_2 μ _Fuc) comprising constitutive transcriptional units for LAC12 from
10 *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and one or two selected fucosyltransferase(s) (see Table 30), and with a second yeast expression plasmid (a pRS420-plasmid variant) comprising constitutive transcriptional units for the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, neuB from *N. meningitidis* with SEQ ID NO 18, neuA from *P. multocida* with SEQ ID NO 22 and one
15 or two selected sialyltransferase(s) (see Table 30), and with a third yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N. meningitidis* with SEQ ID NO 27 and WbgO from *E. coli* O55:H7 with SEQ ID NO 28. The mutant yeast strains is evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LNB, sialylated LNB, LN3, sialylated LN3, LNT and fucosylated and sialylated LNT structures (Table 32) in a growth
20 experiment according to the culture conditions in Example 2 using SD CSM-Ura-Trp-His drop-out medium comprising lactose as precursor.

Table 32: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *S. cerevisiae* strains expressing selected fucosyltransferase and sialyltransferase genes
25 and cultivated in SD CSM-Ura-Trp-His drop-out medium comprising lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SY10	pYF_1C, pYS_1A	2'FL, DiFL, 3'SL, 3'S-2'FL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa, 3'SLNB
SY11	pYF_1D, pYS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LSTa, 3'SLNB
SY12	pYF_1C, pYS_1B	2'FL, DiFL, 6'SL, 6'S-2'FL, LN3, 6'S-LN3, LNT, LNFP-I, 6'SLNB
SY13	pYF_1D, pYS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, LNT, 6'S-LN3, 6'SLNB
SY14	pYF_1C, pYS_2	2'FL, DiFL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa, 3'SLNB, 6'SLNB
SY15	pYF_1D, pYS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LSTa, 3'SLNB, 6'SLNB
SY16	pYF_2CD, pYS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNT, LNFP-I, LSTa, 3'SLNB

SY17	pYF_2CD, pYS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNT, LNFP-I, 6'SLNB
SY18	pYF_2CD, pYS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNT, LNFP-I, LSTa, 3'SLNB, 6'SLNB

*See Table 30 for plasmid info

Example 92. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *S. cerevisiae* host

- 5 An *S. cerevisiae* strain is adapted for production of GDP-fucose, CMP-sialic acid and LNnT and for expression of selected fucosyltransferases and sialyltransferases as described in Example 2 with a first yeast expression plasmid (a variant of p2a_2μ_Fuc) comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12 and one or two selected fucosyltransferase(s) (see Table 30), and with a second yeast expression plasmid
- 10 (a pRS420-plasmid variant) comprising constitutive transcriptional units for the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, neuB from *N. meningitidis* with SEQ ID NO 18, neuA from *P. multocida* with SEQ ID NO 22 and one or two selected sialyltransferase(s) (see Table 30), and with a third yeast expression plasmid comprising constitutive transcriptional units for galE from *E. coli* with SEQ ID NO 30, LgtA from *N.*
- 15 *meningitidis* with SEQ ID NO 27 and LgtB from *N. meningitidis* with SEQ ID NO 29. The mutant yeast strain is evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose, LacNAc, sialylated LacNAc, LN3, sialylated LN3, LNnT, and fucosylated and sialylated LNnT structures (Table 33) in a growth experiment according to the culture conditions in Example 2 using SD CSM-Ura-Trp-His drop-out medium comprising lactose as precursor.

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Table 33: Evaluation of production of a mixture comprising tri-, tetra- and penta-oligosaccharides in whole broth of mutant *S. cerevisiae* strains expressing selected fucosyltransferase and sialyltransferase genes and cultivated in SD CSM-Ura-Trp-His drop-out medium comprising lactose as precursor.

Strain	Plasmids* present	Oligosaccharides
SY19	pYF_1C, pYS_1A	2'FL, DiFL, 3'SL, 3'S-2'FL, LN3, 3'S-LN3, LNnT, LSTd, 3'SLacNAc
SY20	pYF_1D, pYS_1A	3-FL, 3'SL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III, LSTd, 3'SLacNAc
SY21	pYF_1C, pYS_1B	2'FL, DiFL, 6'SL, 6'S-2'FL, LN3, 6'S-LN3, LNnT, LSTc, 6'SLacNAc
SY22	pYF_1D, pYS_1B	3-FL, 6'SL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, 6'SLacNAc
SY23	pYF_1C, pYS_2	2'FL, DiFL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LSTc, LSTd, 3'SLacNAc, 6'SLacNAc
SY24	pYF_1D, pYS_2	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd, 3'SLacNAc, 6'SLacNAc
SY25	pYF_2CD, pYS_1A	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL, LN3, 3'S-LN3, LNnT, LNFP-III,

		LSTd, 3'SLacNAc
SY26	pYF_2CD, pYS_1B	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL, LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, 6'SLacNAc
SY27	pYF_2CD, pYS_2	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL, LN3, 3'S-LN3, 6'S-LN3, LNnT, LNFP-III, LSTc, LSTd, 3'SLacNAc, 6'SLacNAc

*See Table 30 for plasmid info

Example 93. Production of a mammalian milk oligosaccharide mixture comprising fucosylated and sialylated oligosaccharide structures with a modified *S. cerevisiae* host

- 5 An *S. cerevisiae* strain is adapted for production of GDP-fucose and CMP-sialic acid and for expression of one or more fucosyltransferases and one or more sialyltransferases with a yeast artificial chromosome (YAC) comprising constitutive transcriptional units for LAC12 from *K. lactis* with SEQ ID NO 31, gmd from *E. coli* with SEQ ID NO 11, fcl from *E. coli* with SEQ ID NO 12, the mutant glmS*54 from *E. coli* with SEQ ID NO 19, the phosphatase yqaB from *E. coli* with SEQ ID NO 20, AGE from *B. ovatus* with SEQ ID NO 17, neuB
- 10 from *N. meningitidis* with SEQ ID NO 18, neuA from *P. multocida* with SEQ ID NO 22, one or two selected fucosyltransferase(s) and one or two selected sialyltransferases(s). Table 34 shows the fucosyltransferases and sialyltransferases selected in the YACs created in this experiment. The mutant yeast strains are evaluated for production of an oligosaccharide mixture comprising fucosylated and sialylated lactose structures, as shown in Table 35, in a growth experiment according to the culture conditions in Example
- 15 2 using SD CSM medium comprising lactose as precursor.

Table 34: Overview of the fucosyltransferase gene(s) and sialyltransferase gene(s) cloned in the different yeast artificial chromosomes

Yeast artificial chromosomes	Fucosyltransferase(s)		Sialyltransferase(s)	
	a1,2- linkage	a1,3-linkage	a2,3-linkage	a2,6-linkage
YAC1	SEQ ID NO 04	None	SEQ ID NO 23	None
YAC2	None	SEQ ID NO 05	SEQ ID NO 23	None
YAC3	SEQ ID NO 04	None	None	SEQ ID NO 25
YAC4	None	SEQ ID NO 05	None	SEQ ID NO 25
YAC5	SEQ ID NO 04	None	SEQ ID NO 23	SEQ ID NO 25
YAC6	None	SEQ ID NO 05	SEQ ID NO 23	SEQ ID NO 25
YAC7	SEQ ID NO 04	SEQ ID NO 05	SEQ ID NO 23	None
YAC8	SEQ ID NO 04	SEQ ID NO 05	None	SEQ ID NO 25
YAC9	SEQ ID NO 04	SEQ ID NO 05	SEQ ID NO 23	SEQ ID NO 25

Table 35: Evaluation of oligosaccharide production in whole broth of mutant *S. cerevisiae* strains expressing selected fucosyltransferase and sialyltransferase genes from yeast artificial chromosomes and cultivated in SD CSM medium comprising lactose as precursor.

Strain	YACs* present	Oligosaccharides
SY28	YAC1	2'FL, 3'SL, 3'S-2'FL
SY29	YAC2	3-FL, 3'SL, 3'S-3-FL
SY30	YAC3	2'FL, 6'SL, 6'S-2'FL
SY31	YAC4	3-FL, 6'SL, 6'S-3-FL
SY32	YAC5	2'FL, 3'SL, 3'S-2'FL, 6'SL, 6'S-2'FL
SY33	YAC6	3-FL, 3'SL, 3'S-3-FL, 6'SL, 6'S-3-FL
SY34	YAC7	2'FL, 3-FL, DiFL, 3'SL, 3'S-2'FL, 3'S-3-FL
SY35	YAC8	2'FL, 3-FL, DiFL, 6'SL, 6'S-2'FL, 6'S-3-FL
SY36	YAC9	2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL

*See Table 34 for the YAC overview

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Example 94. Material and methods *Bacillus subtilis*

Media

Two different media are used, namely a rich Luria Broth (LB) and a minimal medium for shake flask (MMsf). The minimal medium uses a trace element mix.

10 Trace element mix consisted of 0.735 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g/L $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.033 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17 g/L ZnCl_2 , 0.0311 g/L H_3BO_4 , 0.4 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 0.06 g/L Na_2MoO_4 . The Fe-citrate solution contained 0.135 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1 g/L Na-citrate (Hoch 1973 PMC1212887).

The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). Luria Broth agar (LBA) plates
15 consisted of the LB media, with 12 g/L agar (Difco, Erembodegem, Belgium) added.

The minimal medium for the shake flasks (MMsf) experiments contained 2.00 g/L $(\text{NH}_4)_2\text{SO}_4$, 7.5 g/L KH_2PO_4 , 17.5 g/L K_2HPO_4 , 1.25 g/L Na-citrate, 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L tryptophan, from 10 up to 30 g/L glucose or another carbon source including but not limited to fructose, maltose, sucrose, glycerol and maltotriose when specified in the examples, 10 ml/L trace element mix and 10 ml/L Fe-citrate
20 solution. The medium was set to a pH of 7 with 1M KOH. Depending on the experiment lactose, LNB or LacNAc could be added.

Complex medium, e.g. LB, was sterilized by autoclaving (121°C, 21') and minimal medium by filtration (0.22 μm Sartorius). When necessary, the medium was made selective by adding an antibiotic (e.g. zeocin (20mg/L)).

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Strains, plasmids and mutations

Bacillus subtilis 168, available at Bacillus Genetic Stock Center (Ohio, USA).

Plasmids for gene deletion via Cre/lox are constructed as described by Yan et al. (Appl. & Environm. Microbial., Sept 2008, p5556-5562). Gene disruption is done via homologous recombination with linear DNA and transformation via electroporation as described by Xue et al. (J. Microb. Meth. 34 (1999) 183-191). The method of gene knockouts is described by Liu et al. (Metab. Engine. 24 (2014) 61-69). This method uses 1000bp homologies up- and downstream of the target gene.

Integrative vectors as described by Popp et al. (Sci. Rep., 2017, 7, 15158) are used as expression vector and could be further used for genomic integrations if necessary. A suitable promoter for expression can be derived from the part repository (iGem): sequence id: Bba_K143012, Bba_K823000, Bba_K823002 or Bba_K823003. Cloning can be performed using Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation.

In an example for the production of lactose-based oligosaccharides, *Bacillus subtilis* mutant strains are created to contain a gene coding for a lactose importer (such as the *E. coli* lacY with SEQ ID NO 15). In an example for 2'FL, 3FL and/or diFL production, an alpha-1,2- and/or alpha-1,3-fucosyltransferase expression construct is additionally added to the strains. In an example for LN3 production, a constitutive transcriptional unit comprising a galactoside beta-1,3-N-acetylglucosaminyltransferase like e.g. lgtA from *N. meningitidis* (SEQ ID NO 27) is additionally added to the strain. In an example for LNT production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,3-galactosyltransferase like e.g. WbgO from *E. coli* O55:H7 (SEQ ID NO 28). In an example for LNNt production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,4-galactosyltransferase like e.g. lgtB from *N. meningitidis* (SEQ ID NO 29).

In an example for sialic acid production, a mutant *B. subtilis* strain is created by overexpressing a fructose-6-P-aminotransferase like the native fructose-6-P-aminotransferase (UniProt ID P0CI73) to enhance the intracellular glucosamine-6-phosphate pool. Further on, the enzymatic activities of the genes nagA, nagB and gamA are disrupted by genetic knockouts and a glucosamine-6-P-aminotransferase like e.g. from *S. cerevisiae* (SEQ ID NO 16), an N-acetylglucosamine-2-epimerase like e.g. from *B. ovatus* (SEQ ID NO 17) and an N-acetylneuraminate synthase like e.g. from *N. meningitidis* (SEQ ID NO 18) are overexpressed on the genome. To allow sialylated oligosaccharide production, the sialic acid producing strain is further modified with a constitutive transcriptional unit comprising an N-acylneuraminate cytidylyltransferase like e.g. the NeuA enzyme from *P. multocida* (SEQ ID NO 22), and one or more copies of a beta-galactoside alpha-2,3-sialyltransferase like e.g. PmultST3 from *P. multocida* (UniProt ID Q9CLP3) or a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity (SEQ ID NO 23), or NmeniST3 from *N. meningitidis* (SEQ ID NO 24) or PmultST2 from *P. multocida* subsp. *multocida* str. Pm70 (GenBank No. AAK02592.1), a beta-galactoside

alpha-2,6-sialyltransferase like e.g. PdST6 from *Photobacterium damsela* (UniProt ID O66375) or a PdST6-like polypeptide consisting of amino acid residues 108 to 497 of UniProt ID O66375 having beta-galactoside alpha-2,6-sialyltransferase activity (SEQ ID NO 25) or P-JT-ISH-224-ST6 from *Photobacterium* sp. JT-ISH-224 (UniProt ID A8QYL1) or a P-JT-ISH-224-ST6-like polypeptide consisting of amino acid residues 18 to 514 of UniProt ID A8QYL1 having beta-galactoside alpha-2,6-sialyltransferase activity (SEQ ID NO 26), and/or an alpha-2,8-sialyltransferase like e.g. from *M. musculus* (UniProt ID Q64689).

Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9, Twist Biosciences or IDT.

Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Cultivation conditions

A preculture of 96-well microtiter plate experiments was started from a cryovial or a single colony from an LB plate, in 150 µL LB and was incubated overnight at 37°C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96-well square microtiter plate, with 400 µL MMs medium by diluting 400x. Each strain was grown in multiple wells of the 96-well plate as biological replicates. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer.

At the end of the cultivation experiment samples were taken from each well to measure the supernatant concentration (extracellular sugar concentrations, after 5 min. spinning down the cells), or by boiling the culture broth for 15 min at 90°C or for 60 min at 60°C before spinning down the cells (= whole broth concentration, intra- and extracellular sugar concentrations, as defined herein).

Also, a dilution of the cultures was made to measure the optical density at 600 nm. The cell performance index or CPI was determined by dividing the oligosaccharide concentrations by the biomass, in relative percentages compared to a reference strain. The biomass is empirically determined to be approximately 1/3rd of the optical density measured at 600 nm.

Example 95. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL with a modified *B. subtilis* host

A *B. subtilis* strain is modified as described in Example 94 by genomic knock-ins of constitutive transcriptional units for the lactose permease (LacY) from *E. coli* with SEQ ID NO 15 and the alpha-1,2-fucosyltransferase HpFutC with SEQ ID NO 04 and the alpha-1,3-fucosyltransferase HpFucT with SEQ ID NO 05. The novel strain is evaluated for the production of 2'FL, 3-FL and DiFL in a growth experiment on MMs medium comprising lactose according to the culture conditions provided in Example 94. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 96. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, DiFL, LNFP-I, LNT and LN3 with a modified *B. subtilis* host

A *B. subtilis* strain is first modified for LN3 production and growth on sucrose by genomic knock-out of the *nagB*, *glmS* and *gamA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID P0CI73), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* (SEQ ID NO 27), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the N-acetylglucosamine beta-1,3-galactosyltransferase WbgO from *E. coli* O55:H7 (SEQ ID NO 28) to produce LNT. In a subsequent step, the LNT producing strain is transformed with an expression plasmid comprising a constitutive transcriptional unit for the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04). The novel strain is evaluated for the production of an oligosaccharide mixture comprising 2'FL, DiFL, LN3, LNT and LNFP-I in a growth experiment on MMs medium comprising lactose as precursor according to the culture conditions provided in Example 94. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 97. Production of a mammalian milk oligosaccharide mixture comprising 3'SL, LN3, LNT, sialylated LN3, LSTa with a modified *B. subtilis* host

The mutant *B. subtilis* strain producing LNT as described in Example 96 is further modified with a genomic knock-out of the *nagA* gene and a second compatible expression plasmid comprising the TRP1 selection marker and constitutive transcriptional units for two copies of the mutant L-glutamine—D-fructose-6-phosphate aminotransferase (*glmS**54) from *E. coli* (SEQ ID NO 19), a phosphatase like e.g. a phosphatase chosen from the *E. coli* genes comprising *aphA*, *Cof*, *HisB*, *OtsB*, *SurE*, *Yaed*, *YcjU*, *YedP*, *YfbT*, *YidA*, *YigB*, *YihX*, *YniC*, *YqaB*, *YrbL*, *AppA*, *Gph*, *SerB*, *YbhA*, *YbiV*, *YbjL*, *Yfb*, *YieH*, *YjgL*, *YjjG*, *YrfG* and *YbiU* or *PsMupP* from *P. putida*, *ScDOG1* from *S. cerevisiae* or *BsAraL* from *B. subtilis* as described in WO18122225, the N-acetylglucosamine 2-epimerase (AGE) from *B. ovatus* (SEQ ID NO 17), the N-acetylneuraminate synthase (NeuB) from *N. meningitidis* (SEQ ID NO 18), the N-acylneuraminate cytidyltransferase NeuA from *P. multocida* (SEQ ID NO 22) and three copies of a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity like SEQ ID NO 23. The novel strain is evaluated for the production of a mixture comprising 3'SL, LN3, sialylated LN3, LNT, and LSTa (Neu5Ac-a2,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc) in a growth experiment on MMs medium comprising lactose as precursor according to the culture conditions provided in Example 94. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 98. Production of an oligosaccharide mixture comprising 2'FL, DiFL, LNFP-I, LNFP-II, LNT and LN3 with a modified *B. subtilis* host

A *B. subtilis* strain is first modified for LN3 production and growth on sucrose by genomic knock-out of the *nagB*, *glmS* and *gamA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID P0CI73), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* (SEQ ID NO 27), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the N-acetylglucosamine beta-1,3-galactosyltransferase WbgO from *E. coli* O55:H7 (SEQ ID NO 28) to produce LNT. In a subsequent step, the LNT producing strain is transformed with an expression plasmid comprising constitutive transcriptional units for the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04) and a mutant a1,3/4 fucosidase from *B. longum* subsp. *infantis* ATCC 15697 with SEQ ID NO 39. The novel strain is evaluated for the production of an oligosaccharide mixture comprising LN3, LNT, LNFP-I, LNFP-II, 2'FL and DiFL in a growth experiment on MMSf medium comprising lactose as precursor according to the culture conditions provided in Example 94. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 99. Production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL with a modified *B. subtilis* host

A *B. subtilis* strain is modified by genomic knock-out of the *nagA*, *nagB*, *glmS* and *gamA* genes and genomic knock-ins of constitutive transcriptional units for the lactose permease (LacY) from *E. coli* with SEQ ID NO 15, the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02), the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03), the native fructose-6-P-aminotransferase (UniProt ID P0CI73), the glucosamine 6-phosphate N-acetyltransferase GNA1 from *S. cerevisiae* (SEQ ID NO 16), the mutant L-glutamine—D-fructose-6-phosphate aminotransferase (*glmS**54) from *E. coli* (SEQ ID NO 19), a phosphatase like e.g. a phosphatase chosen from the *E. coli* genes comprising *aphA*, *Cof*, *HisB*, *OtsB*, *SurE*, *Yaed*, *YcjU*, *YedP*, *YfbT*, *YidA*, *YigB*, *YihX*, *YniC*, *YqaB*, *YrbL*, *AppA*, *Gph*, *SerB*, *YbhA*, *YbiV*, *YbjL*, *Yfb*, *YieH*, *YjgL*, *YjjG*, *YrfG* and *YbiU* or *PsMupP* from *P. putida*, *ScDOG1* from *S. cerevisiae* or *BsAraL* from *B. subtilis* as described in WO18122225, the N-acetylglucosamine 2-epimerase (AGE) from *B. ovatus* (SEQ ID NO 17), the N-acetylneuraminate synthase (NeuB) from *N. meningitidis* (SEQ ID NO 18) and the N-acylneuraminate cytidyltransferase NeuA from *P. multocida* (SEQ ID NO 22). In a next step, the strain is transformed with an expression plasmid comprising constitutive transcriptional units for three copies of a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity like

SEQ ID NO 23 and three copies of a PdST6-like polypeptide consisting of amino acid residues 108 to 497 of UniProt ID O66375 having beta-galactoside alpha-2,6-sialyltransferase activity (SEQ ID NO 25). In a further step, the mutant strain is transformed with a second compatible expression plasmid comprising constitutive transcriptional units for the alpha-1,2-fucosyltransferase HpFutC with SEQ ID NO 04 and the alpha-1,3-fucosyltransferase HpFucT with SEQ ID NO 05. The novel strain is evaluated for the production of 2'FL, 3-FL, DiFL, 3'SL, 6'SL, 3'S-2'FL, 3'S-3-FL, 6'S-2'FL, 6'S-3-FL in a growth experiment on MMs medium comprising lactose according to the culture conditions provided in Example 94. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

10 Example 100. Production of an oligosaccharide mixture comprising LN3, LNnT, GalNAc-b1,3-LNnT, Gal-b1,3-GalNAc-b1,3-LNnT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose and (GalNAc)-poly-LNnT with a modified *B. subtilis* strain

A *B. subtilis* strain is first modified by genomic knock-out of the *lacZ*, *nagB*, *gamA*, *glk*, *galE*, *galT*, *galK* and *galM* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID P0CI73), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). The thus obtained mutant strain is further modified with genomic knock-ins of constitutive transcriptional units comprising *lgtB* from *N. meningitidis* (SEQ ID NO 29), *galE* from *E. coli* (SEQ ID NO 30), *lgtA* from *N. meningitidis* (SEQ ID NO 27), the mutant *glmS**54 from *E. coli* (SEQ ID NO 19), *WbpP* from *P. aeruginosa* (SEQ ID NO 34) and *LgtD* from *H. influenzae* (SEQ ID NO 35). The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, LNnT, GalNAc-b1,3-LNnT, Gal-b1,3-GalNAc-b1,3-LNnT, GalNAc-b1,3-lactose and Gal-b1,3-GalNAc-b1,3-lactose as well as poly-LNnT structures and GalNAc-ylated poly-LNnT structures in a growth experiment on MMs medium comprising lactose as precursor according to the culture conditions provided in Example 94.

30 Example 101. Production of an oligosaccharide mixture comprising LacNAc, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, LN3, LNnT, GalNAc-b1,3-LacNAc, Gal-b1,3-GalNAc-b1,3-LacNAc, poly-LacNAc (Gal-b1,4-GlcNAc)_n and GalNAc-ylated poly-LacNAc structures with a modified *B. subtilis* strain

A *B. subtilis* strain is first modified by genomic knock-out of the *lacZ*, *nagB*, *gamA*, *glk*, *galE*, *galT*, *galK* and *galM* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID P0CI73), the mutant *glmS**54 from *E. coli* (SEQ ID NO 19), *GNA1* from *S. cerevisiae* (SEQ ID NO 16), the phosphatase *yqaB* from *E. coli* (SEQ ID NO 20), the galactoside beta-1,3-N-acetylglucosaminyltransferase (*LgtA*) from *N. meningitidis* (SEQ ID NO 27), *LgtB* from *N. meningitidis* (SEQ ID NO 29), the 4-epimerase (*WbpP*) of *Pseudomonas aeruginosa* (SEQ ID NO 34) and the β 1,3-N-acetylgalactosaminyltransferase

(LgtD) from *Haemophilus influenzae* (SEQ ID NO 35). The novel strain is evaluated for production of an oligosaccharide mixture comprising LacNAc, LN3, LNNt, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc, GalNAc-b1,3-LacNAc, Gal-b1,3-GalNAc-b1,3-LacNAc, poly-LacNAc structures, i.e. (Gal-b1,4-GlcNAc)_n and GalNAc-ylated poly-LacNAc structures, in a growth experiment on MMSf medium comprising lactose as precursor according to the culture conditions provided in Example 94.

Example 102. Material and methods *Corynebacterium glutamicum*

Media

Two different media are used, namely a rich tryptone-yeast extract (TY) medium and a minimal medium for shake flask (MMSf). The minimal medium uses a 1000x stock trace element mix.

Trace element mix consisted of 10 g/L CaCl₂, 10 g/L FeSO₄·7H₂O, 10 g/L MnSO₄·H₂O, 1 g/L ZnSO₄·7H₂O, 0.2 g/L CuSO₄, 0.02 g/L NiCl₂·6H₂O, 0.2 g/L biotin (pH 7.0) and 0.03 g/L protocatechuic acid.

The minimal medium for the shake flasks (MMSf) experiments contained 20 g/L (NH₄)₂SO₄, 5 g/L urea, 1 g/L KH₂PO₄, 1 g/L K₂HPO₄, 0.25 g/L MgSO₄·7H₂O, 42 g/L MOPS, from 10 up to 30 g/L glucose or another carbon source including but not limited to fructose, maltose, sucrose, glycerol and maltotriose when specified in the examples and 1 ml/L trace element mix. Depending on the experiment lactose, LNB, and/or LacNAc could be added to the medium.

The TY medium consisted of 1.6% tryptone (Difco, Erembodegem, Belgium), 1% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). TY agar (TYA) plates consisted of the TY media, with 12 g/L agar (Difco, Erembodegem, Belgium) added.

Complex medium, e.g. TY, was sterilized by autoclaving (121°C, 21') and minimal medium by filtration (0.22 µm Sartorius). When necessary, the medium was made selective by adding an antibiotic (e.g., kanamycin, ampicillin).

Strains and mutations

Corynebacterium glutamicum ATCC 13032, available at the American Type Culture Collection.

Integrative plasmid vectors based on the Cre/loxP technique as described by Suzuki et al. (Appl. Microbiol. Biotechnol., 2005 Apr, 67(2):225-33) and temperature-sensitive shuttle vectors as described by Okibe et al. (Journal of Microbiological Methods 85, 2011, 155-163) are constructed for gene deletions, mutations and insertions. Suitable promoters for (heterologous) gene expression can be derived from Yim et al. (Biotechnol. Bioeng., 2013 Nov, 110(11):2959-69). Cloning can be performed using Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation.

In an example for the production of lactose-based oligosaccharides, *C. glutamicum* mutant strains are created to contain a gene coding for a lactose importer (such as e.g. the E. coli lacY with SEQ ID NO 15). In an example for 2'FL, 3FL and/or diFL production, an alpha-1,2- and/or alpha-1,3-fucosyltransferase

expression construct is additionally added to the strains.

In an example for LN3 production, a constitutive transcriptional unit comprising a galactoside beta-1,3-N-acetylglucosaminyltransferase like e.g. lgtA from *N. meningitidis* (SEQ ID NO 27) is additionally added to the strain. In an example for LNT production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,3-galactosyltransferase like e.g. WbgO from *E. coli* O55:H7 (SEQ ID NO 28). In an example for LNnT production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,4-galactosyltransferase like e.g. lgtB from *N. meningitidis* (SEQ ID NO 29).

In an example for sialic acid production, a mutant *C. glutamicum* strain is created by overexpressing a fructose-6-P-aminotransferase like the native fructose-6-P-aminotransferase (UniProt ID Q8NND3) to enhance the intracellular glucosamine-6-phosphate pool. Further on, the enzymatic activities of the genes nagA, nagB and gamA are disrupted by genetic knockouts and a glucosamine-6-P-aminotransferase like e.g. from *S. cerevisiae* (SEQ ID NO 16), an N-acetylglucosamine-2-epimerase like e.g. from *B. ovatus* (SEQ ID NO 17) and an N-acetylneuraminate synthase like e.g. from *N. meningitidis* (SEQ ID NO 18) are overexpressed on the genome. To allow sialylated oligosaccharide production, the sialic acid producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylneuraminate cytidyltransferase like e.g. the NeuA enzyme from *P. multocida* (SEQ ID NO 22), and one or more copies of a beta-galactoside alpha-2,3-sialyltransferase like e.g. PmultST3 from *P. multocida* (UniProt ID Q9CLP3) or a PmultST3-like polypeptide consisting of amino acid residues 1 to 268 of UniProt ID Q9CLP3 having beta-galactoside alpha-2,3-sialyltransferase activity (SEQ ID NO 23), or NmeniST3 from *N. meningitidis* (SEQ ID NO 24) or PmultST2 from *P. multocida* subsp. *multocida* str. Pm70 (GenBank No. AAK02592.1), a beta-galactoside alpha-2,6-sialyltransferase like e.g. PdST6 from *Photobacterium damsela* (UniProt ID O66375) or a PdST6-like polypeptide consisting of amino acid residues 108 to 497 of UniProt ID O66375 having beta-galactoside alpha-2,6-sialyltransferase activity (SEQ ID NO 25) or P-JT-ISH-224-ST6 from *Photobacterium* sp. JT-ISH-224 (UniProt ID A8QYL1) or a P-JT-ISH-224-ST6-like polypeptide consisting of amino acid residues 18 to 514 of UniProt ID A8QYL1 having beta-galactoside alpha-2,6-sialyltransferase activity (SEQ ID NO 26), and/or an alpha-2,8-sialyltransferase like e.g. from *M. musculus* (UniProt ID Q64689).

Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9, Twist Biosciences or IDT.

Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Cultivation conditions

A preculture of 96-well microtiter plate experiments was started from a cryovial or a single colony from a TY plate, in 150 µL TY and was incubated overnight at 37°C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96-well square microtiter plate, with 400 µL MMs medium by diluting 400x.

Each strain was grown in multiple wells of the 96-well plate as biological replicates. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer. At the end of the cultivation experiment samples were taken from each well to measure the supernatant concentration (extracellular sugar concentrations, after 5 min. spinning down the cells), or by boiling the culture broth for 15 min at 60°C before spinning down the cells (= whole broth concentration, intra- and extracellular sugar concentrations, as defined herein).

Also, a dilution of the cultures was made to measure the optical density at 600 nm. The cell performance index or CPI was determined by dividing the oligosaccharide concentrations, measured in the whole broth by the biomass, in relative percentages compared to the reference strain. The biomass is empirically determined to be approximately 1/3rd of the optical density measured at 600 nm.

Example 103. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 3'SL and 6'SL with a modified *C. glutamicum* host

A *C. glutamicum* strain is modified as described in Example 102 by genomic knockouts of the *C. glutamicum* genes *ldh*, *cgl2645*, *nagB*, *gamA* and *nagA*, together with genomic knock-ins of constitutive transcriptional units for the lactose permease (LacY) from *E. coli* with SEQ ID NO 15, the sucrose permease CscB from *E. coli* W with SEQ ID NO 01, the fructose kinase Frk originating from *Zymomonas mobilis* with SEQ ID NO 02, the sucrose phosphorylase BaSP originating from *Bifidobacterium adolescentis* with SEQ ID NO 03, the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), the glucosamine-6-P-aminotransferase from *S. cerevisiae* (SEQ ID NO 16), the N-acetylglucosamine-2-epimerase from *B. ovatus* (SEQ ID NO 17), and the N-acetylneuraminate synthase from *N. meningitidis* (SEQ ID NO 18). In a next step, the novel strain is transformed with an expression plasmid comprising constitutive transcriptional units for the NeuA enzyme from *P. multocida* (SEQ ID NO 22), the beta-galactoside alpha-2,3-sialyltransferase PmultST3 from *P. multocida* (UniProt ID Q9CLP3), the beta-galactoside alpha-2,6-sialyltransferase PdST6 from *P. damsela* (UniProt ID O66375), the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04) and the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). The novel strain is evaluated for the production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, 3'SL and 6'SL in a growth experiment on MMs medium comprising lactose according to the culture conditions provided in Example 102. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 104. Production of a mammalian milk oligosaccharide mixture comprising 3-FL, LNFP-III, LNnT and LN3 with a modified *C. glutamicum* host

A *C. glutamicum* strain is modified as described in Example 102 for LN3 production and growth on sucrose by genomic knock-out of the *nagB*, *glmS* and *gamA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* (SEQ ID NO 27), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the N-acetylglucosamine beta-1,4-galactosyltransferase LgtB from *N. meningitidis* (SEQ ID NO 29) to produce LNnT. In a subsequent step, the LNnT producing strain is transformed with an expression plasmid comprising a constitutive transcriptional unit for the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). The novel strain is evaluated for the production of an oligosaccharide mixture comprising 3-FL, LN3, LNnT and LNFP-III in a growth experiment on MMsf medium comprising lactose as precursor according to the culture conditions provided in Example 102. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 105. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNnT, LNFP-III and lacto-N-neohexaose with a modified *C. glutamicum* host

A *C. glutamicum* strain is modified for LN3 production and growth on sucrose by genomic knock-out of the *nagB*, *glmS* and *gamA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* (SEQ ID NO 27), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the N-acetylglucosamine beta-1,4-galactosyltransferase LgtB from *N. meningitidis* (SEQ ID NO 29) to produce LNnT. In a subsequent step, the LNnT producing strain is transformed with an expression plasmid comprising a constitutive transcriptional unit for the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04) and the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). The novel strain is evaluated for the production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNnT, LNFP-III and lacto-N-neohexaose in a growth experiment on MMsf medium comprising lactose as precursor according to the culture conditions provided in Example 102. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 106. Production of an oligosaccharide mixture comprising LN3, sialylated LN3, 6'SL, LNnT and LSTc with a modified *C. glutamicum* host

A *C. glutamicum* strain is modified as described in Example 102 for LN3 production and growth on sucrose by genomic knock-out of the *ldh*, *cgl2645*, *nagB*, *gamA* and *nagA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* (SEQ ID NO 27), the sucrose transporter (CscB) from *E. coli* W (SEQ ID NO 01), the fructose kinase (Frk) from *Z. mobilis* (SEQ ID NO 02) and the sucrose phosphorylase (BaSP) from *B. adolescentis* (SEQ ID NO 03). In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the N-acetylglucosamine beta-1,4-galactosyltransferase LgtB from *N. meningitidis* (SEQ ID NO 29) to produce LNnT. In a next step, the mutant strain is further modified with a genomic knock-in of a constitutive transcriptional unit comprising the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), GNA1 from *S. cerevisiae* (SEQ ID NO 16), AGE from *B. ovatus* (SEQ ID NO 17), and the N-acetylneuraminate synthase from *N. meningitidis* (SEQ ID NO 18) to produce sialic acid. In a next step, the novel strain is transformed with an expression plasmid comprising constitutive transcriptional units for the NeuA enzyme from *P. multocida* (SEQ ID NO 22) and the beta-galactoside alpha-2,6-sialyltransferase PdST6 from *P. damsela* (UniProt ID O66375). The novel strain is evaluated for production of an oligosaccharide mixture comprising LN3, 6'-sialylated LN3 (Neu5Ac-a2,6-(GlcNAc-b1,3)-Gal-b1,4-Glc), 6'SL, LNnT and LSTc in a growth experiment on MMSf medium comprising lactose according to the culture conditions provided in Example 102. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

Example 107. Production of an oligosaccharide mixture comprising 3'SL, 6'SL, LNB, 3'-sialylated LNB and 6'-sialylated LNB with a modified *C. glutamicum* host

A *C. glutamicum* strain is modified as described in Example 102 by genomic knock-out of the *ldh*, *cgl2645*, *nagB*, *gamA* and *nagA* genes and genomic knock-ins of constitutive transcriptional units comprising genes encoding the lactose permease (LacY) from *E. coli* (SEQ ID NO 15), WbgO with SEQ ID NO 28 from *E. coli* O55:H7, galE with SEQ ID NO 30 from *E. coli*, the native fructose-6-P-aminotransferase (UniProt ID Q8NND3), glmS*54 with SEQ ID NO 19, the glucosamine-6-P-aminotransferase from *S. cerevisiae* (SEQ ID NO 16), the N-acetylglucosamine-2-epimerase from *B. ovatus* (SEQ ID NO 17), and the N-acetylneuraminate synthase from *N. meningitidis* (SEQ ID NO 18). In a next step, the novel strain is transformed with an expression plasmid comprising constitutive transcriptional units for the NeuA enzyme from *P. multocida* (SEQ ID NO 22), the beta-galactoside alpha-2,3-sialyltransferase PmultST3 from *P. multocida* (UniProt ID Q9CLP3) and the beta-galactoside alpha-2,6-sialyltransferase PdST6 from *P. damsela* (UniProt ID O66375). The novel strain is evaluated for production of an oligosaccharide mixture

comprising 3'SL, 6'SL, LNB, 3'-sialylated LNB (3'SLNB) and 6'-sialylated LNB (6'SLNB) in a growth experiment on MMs medium comprising lactose and glucose according to the culture conditions provided in Example 102. After 72h of incubation, the culture broth is harvested, and the sugars are analysed on UPLC.

5 Example 108. Production of an oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB in mutant *C. glutamicum* strains

A wild-type *C. glutamicum* strain is first modified with genomic knockouts of the *C. glutamicum* genes *ldh*,
 10 *cgl2645*, *nagB*, *gamA* and *nagA*, together with genomic knock-ins of constitutive transcriptional units comprising genes encoding the phosphatase *yqaB* from *E. coli* (SEQ ID NO 20), *GNA1* from *S. cerevisiae* (SEQ ID NO 16) and *WbgO* from *E. coli* O55:H7 (SEQ ID NO 28) to produce LNB. In a next step, the LNB producing is further modified with knock-ins of constitutive expression units for the 4-epimerase (*WbpP*) of *P. aeruginosa* (SEQ ID NO 34), the galactoside beta-1,3-N-acetylglucosaminyltransferase (*LgtA*) from *N.*
 15 *meningitidis* (SEQ ID NO 27) and the β 1,3-N-acetylgalactosaminyltransferase (*LgtD*) from *H. influenzae* (SEQ ID NO 35). The novel strain is evaluated for production of an oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB in a growth experiment on MMs medium comprising lactose as precursor according to the culture conditions provided in Example 102.

20 Example 109. Materials and methods *Chlamydomonas reinhardtii*

Media

C. reinhardtii cells were cultured in Tris-acetate-phosphate (TAP) medium (pH 7.0). The TAP medium uses a 1000x stock Hutner's trace element mix. Hutner's trace element mix consisted of 50 g/L Na₂EDTA.H₂O
 25 (Titriplex III), 22 g/L ZnSO₄.7H₂O, 11.4 g/L H₃BO₃, 5 g/L MnCl₂.4H₂O, 5 g/L FeSO₄.7H₂O, 1.6 g/L CoCl₂.6H₂O, 1.6 g/L CuSO₄.5H₂O and 1.1 g/L (NH₄)₆MoO₃.

The TAP medium contained 2.42 g/L Tris (tris(hydroxymethyl)aminomethane), 25 mg/L salt stock solution, 0.108 g/L K₂HPO₄, 0.054 g/L KH₂PO₄ and 1.0 mL/L glacial acetic acid. The salt stock solution consisted of 15 g/L NH₄Cl, 4 g/L MgSO₄.7H₂O and 2 g/L CaCl₂.2H₂O. As precursor(s) and/or acceptor(s) for saccharide
 30 synthesis, compounds like e.g. galactose, glucose, fructose, fucose, lactose, LacNAc, LNB could be added. Medium was sterilized by autoclaving (121°C, 21'). For stock cultures on agar slants TAP medium was used containing 1% agar (of purified high strength, 1000 g/cm²).

Strains, plasmids and mutations

35 *C. reinhardtii* wild-type strains 21gr (CC-1690, wild-type, mt+), 6145C (CC-1691, wild-type, mt-), CC-125 (137c, wild-type, mt+), CC-124 (137c, wild-type, mt-) as available from Chlamydomonas Resource Center

(<https://www.chlamycollection.org>), University of Minnesota, U.S.A.

Expression plasmids originated from pSI103, as available from Chlamydomonas Resource Center. Cloning can be performed using Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation. Suitable promoters for (heterologous) gene expression can be derived from e.g. Scranton et al. (Algal Res. 2016, 15: 135-142). Targeted gene modification (like gene knock-out or gene replacement) can be carried using the Crispr-Cas technology as described e.g. by Jiang et al. (Eukaryotic Cell 2014, 13(11): 1465-1469).

Transformation via electroporation was performed as described by Wang et al. (Biosci. Rep. 2019, 39: BSR2018210). Cells were grown in liquid TAP medium under constant aeration and continuous light with a light intensity of 8000 Lx until the cell density reached $1.0\text{--}2.0 \times 10^7$ cells/mL. Then, the cells were inoculated into fresh liquid TAP medium in a concentration of 1.0×10^6 cells/mL and grown under continuous light for 18–20 h until the cell density reached 4.0×10^6 cells/mL. Next, cells were collected by centrifugation at 1250 g for 5 min at room temperature, washed and resuspended with pre-chilled liquid TAP medium containing 60 mM sorbitol (Sigma, U.S.A.), and iced for 10 min. Then, 250 μ L of cell suspension (corresponding to 5.0×10^7 cells) were placed into a pre-chilled 0.4 cm electroporation cuvette with 100 ng plasmid DNA (400 ng/mL). Electroporation was performed with 6 pulses of 500 V each having a pulse length of 4 ms and pulse interval time of 100 ms using a BTX ECM830 electroporation apparatus (1575 Ω , 50 μ FD). After electroporation, the cuvette was immediately placed on ice for 10 min. Finally, the cell suspension was transferred into a 50 ml conical centrifuge tube containing 10 mL of fresh liquid TAP medium with 60 mM sorbitol for overnight recovery at dim light by slowly shaking. After overnight recovery, cells were recollected and plated with starch embedding method onto selective 1.5% (w/v) agar-TAP plates containing ampicillin (100 mg/L) or chloramphenicol (100 mg/L). Plates were then incubated at $23 \pm 0.5^\circ\text{C}$ under continuous illumination with a light intensity of 8000 Lx. Cells were analysed 5-7 days later.

In an example for production of UDP-galactose, *C. reinhardtii* cells are modified with transcriptional units comprising the genes encoding a galactokinase like e.g. from *Arabidopsis thaliana* (KIN, UniProt ID Q9SEE5) and an UDP-sugar pyrophosphorylase like e.g. USP from *A. thaliana* (UniProt ID Q9C5I1).

In an example for LN3 production, a constitutive transcriptional unit comprising a galactoside beta-1,3-N-acetylglucosaminyltransferase like e.g. lgtA from *N. meningitidis* (SEQ ID NO 27) is additionally added to the strain. In an example for LNT production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,3-galactosyltransferase like e.g. WbgO from *E. coli* O55:H7 (SEQ ID NO 28). In an example for LNnT production, the LN3 producing strain is further modified with a constitutive transcriptional unit comprising an N-acetylglucosamine beta-1,4-galactosyltransferase like e.g. lgtB from *N. meningitidis* (SEQ ID NO 29).

In an example for production of GDP-fucose, *C. reinhardtii* cells are modified with a transcriptional unit for a GDP-fucose synthase like e.g. from *Arabidopsis thaliana* (GER1, UniProt ID O49213).

In an example for fucosylation, *C. reinhardtii* cells can be modified with an expression plasmid comprising a constitutive transcriptional unit for an alpha-1,2-fucosyltransferase like e.g. HpFutC from *H. pylori* (SEQ ID NO 04) and/or an alpha-1,3-fucosyltransferase like e.g. HpFucT from *H. pylori* (SEQ ID NO 05).

In an example for CMP-sialic acid synthesis, *C. reinhardtii* cells are modified with constitutive transcriptional units for an UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase like e.g. GNE from *Homo sapiens* (UniProt ID Q9Y223) or a mutant form of the human GNE polypeptide comprising the R263L mutation, an N-acylneuraminate-9-phosphate synthetase like e.g. NANS from *Homo sapiens* (UniProt ID Q9NR45) and an N-acylneuraminate cytidyltransferase like e.g. CMAS from *Homo sapiens* (UniProt ID Q8NFW8). In an example for production of sialylated oligosaccharides, *C. reinhardtii* cells are modified with a CMP-sialic acid transporter like e.g. CST from *Mus musculus* (UniProt ID Q61420), and a Golgi-localised sialyltransferase chosen from species like e.g. *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*.

Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9, Twist Biosciences or IDT.

Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Cultivation conditions

Cells of *C. reinhardtii* were cultured in selective TAP-agar plates at 23 +/- 0.5°C under 14/10 h light/dark cycles with a light intensity of 8000 Lx. Cells were analysed after 5 to 7 days of cultivation.

For high-density cultures, cells could be cultivated in closed systems like e.g. vertical or horizontal tube photobioreactors, stirred tank photobioreactors or flat panel photobioreactors as described by Chen et al. (Bioresour. Technol. 2011, 102: 71-81) and Johnson et al. (Biotechnol. Prog. 2018, 34: 811-827).

Example 110. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LacNAc, 2'FLacNAc and 3-FLacNAc in mutant *C. reinhardtii* cells

C. reinhardtii cells are engineered as described in Example 109 for production of UDP-Gal with genomic knock-ins of constitutive transcriptional units comprising the *Arabidopsis thaliana* genes encoding the galactokinase (KIN, UniProt ID Q9SEE5) and the UDP-sugar pyrophosphorylase (USP) (UniProt ID Q9C5I1). In a next step, the cells are modified with genomic knock-ins of constitutive transcriptional units comprising the b1,4-galactosyltransferase LgtB from *N. meningitidis* (SEQ ID NO 29), GDP-fucose synthase from *Arabidopsis thaliana* (GER1, UniProt ID O49213), the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04) and the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LacNAc,

2'FLacNAc and 3-FLacNAc in a cultivation experiment on TAP-agar plates comprising galactose and N-acetylglucosamine as precursors according to the culture conditions provided in Example 109. After 5 days of incubation, the cells are harvested, and the saccharide production is analysed on UPLC.

5 Example 111. Production of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LacNAc, 2'FLacNAc, 3-FLacNAc, LN3, LNnT, LNFP-III and difucosyl-lacto-N-neohexaose in mutant *C. reinhardtii* cells

The mutant *C. reinhardtii* cells as described in Example 110 are further adapted with a genomic knock-in of a constitutive transcriptional unit for the galactoside beta-1,3-N-acetylglucosaminyltransferase (IgtA) from *N. meningitidis* with SEQ ID NO 27. The novel strain is evaluated for production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LacNAc, 2'FLacNAc, 3-FLacNAc, LN3, LNnT, LNFP-III and difucosyl-lacto-N-neohexaose in a cultivation experiment on TAP-agar plates comprising galactose and N-acetylglucosamine as precursors according to the culture conditions provided in Example 109. After 5 days of incubation, the cells are harvested, and the saccharide production is analysed on UPLC.

15 Example 112. Production of an oligosaccharide mixture comprising sialylated LNB and sialylated LacNAc structures in mutant *C. reinhardtii* cells

C. reinhardtii cells are engineered as described in Example 109 for production of CMP-sialic acid with genomic knock-ins of constitutive transcriptional units comprising a mutant form of the UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase GNE from *Homo sapiens* (UniProt ID Q9Y223) differing from the native polypeptide with a R263L mutation, the N-acylneuraminate-9-phosphate synthetase NANS from *Homo sapiens* (UniProt ID Q9NR45) and the N-acylneuraminate cytidyltransferase CMAS from *Homo sapiens* (UniProt ID Q8NFW8). In a next step, the cells are modified with genomic knock-ins of constitutive transcriptional units comprising the CMP-sialic acid transporter CST from *Mus musculus* (UniProt ID Q61420), the alpha-2,3-sialyltransferases (UniProt IDs P61943 and E9PSJ1) from *Rattus norvegicus* and the alpha-2,6-sialyltransferase (UniProt ID P13721) from *Rattus norvegicus*. In a final step, the cells are transformed with genomic knock-ins of constitutive transcriptional units comprising the *Arabidopsis thaliana* genes encoding the galactokinase (KIN, UniProt ID Q9SEE5) and the UDP-sugar pyrophosphorylase (USP) (UniProt ID Q9C5I1), together with the N-acetylglucosamine beta-1,3-galactosyltransferase WbgO from *E. coli* O55:H7 with SEQ ID NO 28 and the N-acetylglucosamine beta-1,4-galactosyltransferase LgtB from *N. meningitidis* with SEQ ID NO 29. The novel strain is evaluated for production of an oligosaccharide mixture comprising 3'-sialyllacto-N-biose (3'SLNB), 6'-sialyllacto-N-biose (6'SLNB), 3'-sialyllactosamine (3'SLacNAc) and 6'-sialyllactosamine (6'SLacNAc) in a cultivation experiment on TAP-agar plates comprising galactose, glucose and N-acetylglucosamine as precursors according to the culture conditions provided in Example 109. After 5 days of incubation, the cells are harvested, and the saccharide production is analysed on UPLC.

Example 113. Production of an oligosaccharide mixture comprising GlcNAc-b1,3-Gal-b1,4-GlcNAc, beta-Gal-(1,4)-beta-GlcNAc-(1,3)-[beta-GlcNAc-(1,6)]-beta-Gal-(1,4)-GlcNAc and poly-LacNAc structures in mutant *C. reinhardtii* cells

C. reinhardtii cells are engineered as described in Example 109, comprising genomic knock-ins of constitutive transcriptional units comprising the *Arabidopsis thaliana* genes encoding the galactokinase (KIN, UniProt ID Q9SEE5) and the UDP-sugar pyrophosphorylase (USP) (UniProt ID Q9C5I1), the mutant glmS*54 from *E. coli* (differing from the wild-type glmS (UniProt ID P17169) by an A39T, an R250C and an G472S mutation, the phosphatase yqaB from *E. coli* (UniProt ID NP_417175.1), galE from *E. coli* (UniProt ID P09147), LgtA from *N. meningitidis* (UniProt ID Q9JXQ6), LgtB from *N. meningitidis* (UniProt ID Q51116) and the human N-acetyllactosaminide beta-1,6-N-acetylglucosaminyltransferase GCNT2 (UniProt ID Q8N0V5). The novel strains are evaluated for production of an oligosaccharide mixture comprising GlcNAc-b1,3-Gal-b1,4-GlcNAc, beta-Gal-(1,4)-beta-GlcNAc-(1,3)-[beta-GlcNAc-(1,6)]-beta-Gal-(1,4)-GlcNAc and poly-LacNAc structures in a cultivation experiment on TAP-agar plates comprising galactose and GlcNAc as precursors according to the culture conditions provided in Example 109.

Example 114. Materials and Methods animal cells

Isolation of mesenchymal stem cells from adipose tissue of different mammals

Fresh adipose tissue is obtained from slaughterhouses (e.g. cattle, pigs, sheep, chicken, ducks, catfish, snake, frogs) or liposuction (e.g., in case of humans, after informed consent) and kept in phosphate buffer saline supplemented with antibiotics. Enzymatic digestion of the adipose tissue is performed followed by centrifugation to isolate mesenchymal stem cells. The isolated mesenchymal stem cells are transferred to cell culture flasks and grown under standard growth conditions, e.g., 37° C, 5% CO₂. The initial culture medium includes DMEM-F12, RPMI, and Alpha-MEM medium (supplemented with 15% fetal bovine serum), and 1% antibiotics. The culture medium is subsequently replaced with 10% FBS (fetal bovine serum)-supplemented media after the first passage. For example, Ahmad and Shakoory (2013, Stem Cell Regen Med. 9(2): 29- 36), which is incorporated herein by reference in its entirety for all purposes, describes certain variation(s) of the method(s) described herein in this example.

Isolation of mesenchymal stem cells from milk

This example illustrates isolation of mesenchymal stem cells from milk collected under aseptic conditions from human or any other mammal(s) such as described herein. An equal volume of phosphate buffer saline is added to diluted milk, followed by centrifugation for 20 min. The cell pellet is washed thrice with phosphate buffer saline and cells are seeded in cell culture flasks in DMEM-F12, RPMI, and Alpha-MEM medium supplemented with 10% fetal bovine serum and 1% antibiotics under standard culture conditions. For example, Hassiotou et al. (2012, Stem Cells. 30(10): 2164-2174), which is incorporated herein by reference in its entirety for all purposes, describes certain variation(s) of the method(s)

described herein in this example.

Differentiation of stem cells using 2D and 3D culture systems

The isolated mesenchymal cells can be differentiated into mammary-like epithelial and luminal cells in 2D and 3D culture systems. See, for example, Huynh et al. 1991. Exp Cell Res. 197(2): 191 -199; Gibson et al. 1991, In Vitro Cell Dev Biol Anim. 27(7): 585-594; Blatchford et al. 1999; Animal Cell Technology': Basic & Applied Aspects, Springer, Dordrecht. 141-145; Williams et al. 2009, Breast Cancer Res 11(3): 26-43; and Arevalo et al. 2015, Am J Physiol Cell Physiol. 310(5): C348 - C356; each of which is incorporated herein by reference in their entireties for all purposes.

For 2D culture, the isolated cells were initially seeded in culture plates in growth media supplemented with 10 ng/ml epithelial growth factor and 5 pg/ml insulin. At confluence, cells were fed with growth medium supplemented with 2% fetal bovine serum, 1% penicillin-streptomycin (100 U/ml penicillin, 100 ug/ml streptomycin), and 5 pg/ml insulin for 48h. To induce differentiation, the cells were fed with complete growth medium containing 5 pg/ml insulin, 1 pg/ml hydrocortisone, 0.65 ng/ml triiodothyronine, 100 nM dexamethasone, and 1 pg/ml prolactin. After 24h, serum is removed from the complete induction medium.

For 3D culture, the isolated cells were trypsinized and cultured in Matrigel, hyaluronic acid, or ultra- low attachment surface culture plates for six days and induced to differentiate and lactate by adding growth media supplemented with 10 ng/ml epithelial growth factor and 5 pg/ml insulin. At confluence, cells were fed with growth medium supplemented with 2% fetal bovine serum, 1% penicillin-streptomycin (100 U/ml penicillin, 100 ug/ml streptomycin), and 5 pg/ml insulin for 48h. To induce differentiation, the cells were fed with complete growth medium containing 5 pg/ml insulin, 1 pg/ml hydrocortisone, 0.65 ng/ml triiodothyronine, 100 nM dexamethasone, and 1 pg/ml prolactin. After 24h, serum is removed from the complete induction medium.

Method of making mammary-like cells

Mammalian cells are brought to induced pluripotency by reprogramming with viral vectors encoding for Oct4, Sox2, Klf4, and c-Myc. The resultant reprogrammed cells are then cultured in Mammocult media (available from Stem Cell Technologies), or mammary cell enrichment media (DMEM, 3% FBS, estrogen, progesterone, heparin, hydrocortisone, insulin, EGF) to make them mammary-like, from which expression of select milk components can be induced. Alternatively, epigenetic remodelling is performed using remodelling systems such as CRISPR/Cas9, to activate select genes of interest, such as casein, a-lactalbumin to be constitutively on, to allow for the expression of their respective proteins, and/or to down-regulate and/or knock-out select endogenous genes as described e.g. in WO21067641, which is incorporated herein by reference in its entirety for all purposes.

Cultivation

Completed growth media includes high glucose DMEM/F12, 10% FBS, 1% NEAA, 1% pen/strep, 1% ITS-X, 1% F-Glu, 10 ng/ml EGF, and 5 pg/ml hydrocortisone. Completed lactation media includes high glucose DMEM/F12, 1% NEAA, 1% pen/strep, 1% ITS-X, 1% F-Glu, 10 ng/ml EGF, 5 pg/ml hydrocortisone, and 1 pg/ml prolactin (5ug/ml in Hyunh 1991). Cells are seeded at a density of 20,000 cells/cm² onto collagen coated flasks in completed growth media and left to adhere and expand for 48 hours in completed growth media, after which the media is switched out for completed lactation media. Upon exposure to the lactation media, the cells start to differentiate and stop growing. Within about a week, the cells start secreting lactation product(s) such as milk lipids, lactose, casein and whey into the media. A desired concentration of the lactation media can be achieved by concentration or dilution by ultrafiltration. A desired salt balance of the lactation media can be achieved by dialysis, for example, to remove unwanted metabolic products from the media. Hormones and other growth factors used can be selectively extracted by resin purification, for example the use of nickel resins to remove His-tagged growth factors, to further reduce the levels of contaminants in the lactated product.

Example 115. Making of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL and DiFL in a non-mammary adult stem cell

Isolated mesenchymal cells and re-programmed into mammary-like cells as described in Example 114 are modified via CRISPR-CAS to over-express the GDP-fucose synthase GFUS from *Homo sapiens* (UniProt ID Q13630), the alpha-1,2-fucosyltransferase HpFutC from *H. pylori* (SEQ ID NO 04) and the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). Cells are seeded at a density of 20,000 cells/cm² onto collagen coated flasks in completed growth media and left to adhere and expand for 48 hours in completed growth media, after which the media is switched out for completed lactation media for about 7 days. After cultivation as described in Example 114, cells are subjected to UPLC to analyze for production of an oligosaccharide mixture comprising 2'FL, 3-FL and DiFL.

Example 116. Making of a mammalian milk oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT and LNFP-I in a non-mammary adult stem cell

Isolated mesenchymal cells and re-programmed into mammary-like cells as described in Example 114 are modified via CRISPR-CAS to over-express the GlcN6P synthase from *Homo sapiens* (UniProt ID Q06210), the glucosamine 6-phosphate N-acetyltransferase from *Homo sapiens* (UniProt ID Q96EK6), the phosphoacetylglucosamine mutase from *Homo sapiens* (UniProt ID O95394), the UDP-N-acetylhexosamine pyrophosphorylase (UniProt ID Q16222), the galactoside beta-1,3-N-acetylglucosaminyltransferase LgtA from *N. meningitidis* with SEQ ID NO 27, the N-acetylglucosamine beta-1,4-galactosyltransferase LgtB from *N. meningitidis* with SEQ ID NO 29, the GDP-fucose synthase GFUS from *Homo sapiens* (UniProt ID Q13630), the alpha-1,2-fucosyltransferase HpFutC from *H. pylori*

(SEQ ID NO 04) and the alpha-1,3-fucosyltransferase HpFucT from *H. pylori* (SEQ ID NO 05). All genes introduced are codon-optimized to the host cells. Cells are seeded at a density of 20,000 cells/cm² onto collagen coated flasks in completed growth media and left to adhere and expand for 48 hours in completed growth media, after which the media is switched out for completed lactation media for about 7 days. After cultivation as described in Example 114, cells are subjected to UPLC to analyze for production of an oligosaccharide mixture comprising 2'FL, 3-FL, DiFL, LN3, LNT and LNFP-I.

Example 117. Evaluation of production of an oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB in a non-mammary adult stem cell

Isolated mesenchymal cells and re-programmed into mammary-like cells as described in Example 114 are modified via CRISPR-CAS to over-express the beta-1,4-galactosyltransferase 1 B4GalT1 from *Homo sapiens* (UniProt ID P15291), the phosphatase ScDOG1 from *S. cerevisiae* (UniProt ID P38774), GNA1 from *S. cerevisiae* (SEQ ID NO 16) and WbgO from *E. coli* O55:H7 (SEQ ID NO 28), the 4-epimerase (WbpP) of *P. aeruginosa* (SEQ ID NO 34), the galactoside beta-1,3-N-acetylglucosaminyltransferase (LgtA) from *N. meningitidis* (SEQ ID NO 27) and the β 1,3-N-acetylgalactosaminyltransferase (LgtD) from *H. influenzae* (SEQ ID NO 35). All genes introduced in the cells are codon-optimized to the host. Cells are seeded at a density of 20,000 cells/cm² onto collagen coated flasks in completed growth media and left to adhere and expand for 48 hours in completed growth media, after which the media is switched out for completed lactation media for about 7 days. After cultivation as described in Example 114, cells are subjected to UPLC and evaluated for production of an oligosaccharide mixture comprising LNB, LN3, LNT, GalNAc-b1,3-lactose, Gal-b1,3-GalNAc-b1,3-lactose, GalNAc-b1,3-Gal-b1,3-GlcNAc-b1,3-Gal-b1,4-Glc, GalNAc-b1,3-LNB and Gal-b1,3-GalNAc-b1,3-LNB.

Claims

1. A metabolically engineered cell producing a mixture of at least three different mammalian milk oligosaccharides, wherein said cell
 - is metabolically engineered for the production of said mixture, and
 - 5 - expresses at least two glycosyltransferases, and
 - is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferase.
2. Cell according to claim 1, wherein said cell is capable to express, preferably expresses at least five glycosyltransferases, preferably at least six, more preferably at least seven glycosyltransferases.
- 10 3. Cell according to any one of claim 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.
4. Cell according to any one of claim 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged
- 15 oligosaccharides.
5. Cell according to any one of claim 1 or 2, wherein said mixture comprises, consists or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.
6. Cell according to any one of claim 1 or 2, wherein said mixture comprises, consists or consists
- 20 essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.
7. Cell according to any one of claim 1 or 2, wherein said mixture comprises, consists or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.
- 25 8. Cell according to any one of claims 1 to 7, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.
9. Cell according to any one of claim 1 to 8, wherein said cell comprises multiple copies of the same coding DNA sequence encoding for one protein.
- 30 10. Cell according to any one of claims 1 to 9, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.
11. Cell according to any one of claims 1 to 10, wherein said cell produces four or more different mammalian milk oligosaccharides.
12. Cell according to any one of claims 1 to 11, wherein any one of said glycosyltransferase is chosen from
- 35 the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-

acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases,

- preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,

- preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,

- preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,

- preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,

- preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,

- preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,

- preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase.

13. Cell according to any one of claims 1 to 12, wherein said cell is modified in the expression or activity of at least one of said glycosyltransferases.

14. Cell according to any one of claims 1 to 13, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).

15. Cell according to any one of claims 1 to 14, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).

16. Cell according to any one of claims 1 to 15, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).

17. Cell according to any one of claims 1 to 16, wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).

18. Cell according to any one of claim 1 to 17, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine

(UDP-GalNAc).

19. Cell according to any one of claim 1 to 18, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine (UDP-ManNAc).

20. Cell according to any one of claims 1 to 19, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose, UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylglucosamine, UDP-N-acetylglucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumovosamine (UDP-L-PneNAc or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose.

21. Cell according to any one of claims 1 to 20, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acetylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, UDP-N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.

22. Cell according to any one of claims 1 to 21, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.

23. Cell according to any one of claims 1 to 22, wherein at least one of said mammalian milk

oligosaccharides is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

- 5 24. Cell according to any one of claims 1 to 23, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.
25. Cell according to any one of claims 1 to 24, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.
- 10 26. Cell according to any one of claims 1 to 25, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.
27. Cell according to any one of claims 1 to 26, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.
- 15 28. Cell according to any one of claims 1 to 27, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the production of any one or more of said oligosaccharides, said precursor(s) being fed to the cell from the cultivation medium.
29. Cell according to any one of claims 1 to 28, wherein said cell is producing at least one precursor for the production of any one of said oligosaccharides.
- 20 30. Cell according to any one of claims 1 to 29, wherein said at least one precursor for the production of any one of said oligosaccharides is completely converted into any one of said oligosaccharides.
31. Cell according to any one of claims 1 to 30, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.
- 25 32. Cell according to any one of claims 1 to 31, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or
 - iv) expression of a heterologous membrane protein,
- 30 wherein said membrane protein is involved in the secretion of any one of said mammalian milk oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.
33. Cell according to any one of claims 1 to 32, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or

35 ii) modified activity of an endogenous membrane protein, and/or

 - iii) expression of a homologous membrane protein, and/or

iv) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the uptake of a precursor and/or acceptor for the synthesis of any one of said mammalian milk oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.

34. Cell according to any one of claim 32 or 33, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β -barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators, preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters, preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.

35. Cell according to any one of claim 32 to 34, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said oligosaccharides.

36. Cell according to any one of claim 1 to 35, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).

37. Cell according to any one of claim 1 to 36, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.

38. Cell according to claim 37, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminatase lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIBAB-Man, EIIC-Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, Icn protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacetyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

39. Cell according to any one of claim 1 to 38, wherein the cell is capable to produce

phosphoenolpyruvate (PEP).

40. Cell according to any one of claim 1 to 39, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.

41. Cell according to any one of claims 1 to 40, wherein any one of said mammalian milk oligosaccharides is a human milk oligosaccharide, preferably all said oligosaccharides are human milk oligosaccharides.

42. A method to produce a mixture of at least three different mammalian milk oligosaccharides by a cell, the method comprising the steps of:

a. providing a cell, preferably a single cell, expressing at least two glycosyltransferases and capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases, and

b. cultivating said cell under conditions permissive to express said glycosyltransferases and to synthesize said nucleotide-sugar(s), resulting in said cell producing at least three different mammalian milk oligosaccharides,

c. preferably, separating at least one of said mammalian milk oligosaccharides from said cultivation, more preferably, separating all of said mammalian milk oligosaccharides from said cultivation.

43. Method according to claim 42, wherein said cell is capable to express, preferably expresses at least five glycosyltransferases, preferably at least six, more preferably at least seven glycosyltransferases.

44. Method according to any one of claim 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.

45. Method according to any one of claim 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged oligosaccharides.

46. Method according to any one of claim 42 or 43, wherein said mixture comprises, consists or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.

47. Method according to any one of claim 42 or 43, wherein said mixture comprises, consists or consists essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.

48. Method according to any one of claim 42 or 43, wherein said mixture comprises, consists or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.

49. Method according to any one of claim 42 to 48, wherein said cell is a metabolically engineered cell according to any one of embodiments 1 to 41.

50. Method according to claim 49, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is

created by a natural inducer.

51. Method according to any one of claim 49 or 50, wherein said cell comprises multiple copies of the same coding DNA sequence encoding for one protein.
52. Method according to any one of claims 42 to 51, wherein said oligosaccharide mixture comprises at
5 least three different oligosaccharides differing in degree of polymerization.
53. Method according to any one of claims 42 to 52, wherein said cell produces four or more different mammalian milk oligosaccharides.
54. Method according to any one of claims 42 to 53, wherein any one of said glycosyltransferases is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases,
10 glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylramnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-N-acetylglucosamine
15 enolpyruvyl transferases and fucosaminyltransferases,
 - preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,
 - preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase,
20 alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,
 - preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,
 - 25 - preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,
 - preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,
 - preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside
30 beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,
 - preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase,
 - preferably, said cell is modified in the expression or activity of at least one of said glycosyltransferases.
- 35 55. Method according to any one of claims 42 to 54, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).

56. Method according to any one of claims 42 to 55, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).
57. Method according to any one of claims 42 to 56, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).
58. Method according to any one of claims 42 to 57, wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).
59. Method according to any one of claims 42 to 58, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine (UDP-GalNAc).
60. Method according to any one of claims 42 to 59, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine (UDP-ManNAc).
61. Method according to any one of claims 42 to 60, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy--L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy--L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylglucosamine, UDP-N-acetylglucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N₃, CMP-Neu4,5Ac₂, CMP-Neu5,7Ac₂, CMP-Neu5,9Ac₂, CMP-Neu5,7(8,9)Ac₂, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.
62. Method according to any one of claims 42 to 61, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acetylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate

lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucosmutase, UDP-N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and
5 UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.

63. Method according to any one of claims 42 to 62, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.

10 64. Method according to any one of claims 42 to 63, wherein said mammalian milk oligosaccharide mixture comprises at least one fucosylated mammalian milk oligosaccharide.

65. Method according to any one of claims 42 to 64, wherein said mammalian milk oligosaccharide mixture comprises at least one sialylated mammalian milk oligosaccharide.

15 66. Method according to any one of claims 42 to 65, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.

67. Method according to any one of claims 42 to 66, wherein said mammalian milk oligosaccharide mixture comprises at least one galactosylated mammalian milk oligosaccharide.

20 68. Method according to any one of claims 42 to 67, wherein said mammalian milk oligosaccharide mixture comprises at least one mammalian milk oligosaccharide that is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

25 69. Method according to any one of claims 42 to 68, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the production of any one or more of said oligosaccharides, said precursor(s) being fed to the cell from the cultivation medium.

30 70. Method according to any one of claims 42 to 69, wherein said cell is producing at least one precursor for the production of any one of said mammalian milk oligosaccharides.

71. Method according to any one of claims 42 to 70, wherein said at least one precursor for the production of any one of said mammalian milk oligosaccharides is completely converted into any one of said mammalian milk oligosaccharides.

35 72. Method according to any one of claims 42 to 71, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.

73. Method according to any one of claims 42 to 72, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or
 - iv) expression of a heterologous membrane protein,
- wherein said membrane protein is involved in the secretion of any one of said mammalian milk oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.
74. Method according to any one of claims 42 to 73, wherein said cell is further genetically modified for
- i) modified expression of an endogenous membrane protein, and/or
 - ii) modified activity of an endogenous membrane protein, and/or
 - iii) expression of a homologous membrane protein, and/or
 - iv) expression of a heterologous membrane protein,
- wherein said membrane protein is involved in the uptake of a precursor and/or acceptor for the synthesis of any one of said mammalian milk oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.
75. Method according to any one of claim 73 or 74, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β -barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators, preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters, preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.
76. Method according to any one of claim 73 to 75, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said oligosaccharides.
77. Method according to any one of claim 42 to 76, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).
78. Method according to any one of claim 42 to 77, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.
79. Method according to claim 78, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate

transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIAB-Man, EIIC-Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, Icn protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

80. Method according to any one of claim 42 to 79, wherein the cell is capable to produce phosphoenolpyruvate (PEP).

81. Method according to any one of claim 42 to 80, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.

82. Method according to any one of claims 42 to 81, wherein any one of said mammalian milk oligosaccharides as a human milk oligosaccharide, preferably all of said mammalian milk oligosaccharides are human milk oligosaccharides.

83. Method according to any one of claim 42 to 82, wherein said conditions comprise:

- use of a culture medium comprising at least one precursor and/or acceptor for the production of any one of said oligosaccharides, and/or
- adding to the culture medium at least one precursor and/or acceptor feed for the production of any one of said oligosaccharides.

84. Method according to any one of claim 42 to 83, the method comprising at least one of the following steps:

- i) Use of a culture medium comprising at least one precursor and/or acceptor;
- ii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed;
- iii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed and

wherein preferably, the pH of said precursor and/or acceptor feed is set between 3 and 7 and wherein preferably, the temperature of said precursor and/or acceptor feed is kept between 20°C and 80°C;

- iv) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- v) Adding at least one precursor and/or acceptor feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein preferably, the pH of said feeding solution is set between 3 and 7 and wherein preferably, the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

85. Method according to any one of claim 42 to 83, the method comprising at least one of the following steps:

- i) Use of a culture medium comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter);
- ii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said lactose feed;
- iii) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m³ (cubic meter), preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than two-fold of the volume of the culture medium before the addition of said lactose feed and wherein preferably the pH of said lactose feed is set between 3 and 7 and wherein preferably the temperature of said lactose feed is kept between 20°C and 80°C;
- iv) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

- v) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L and wherein preferably the pH of said feeding solution is set between 3 and 7 and wherein preferably the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final cultivation.

86. Method according to claim 85, wherein the lactose feed is accomplished by adding lactose from the beginning of the cultivation in a concentration of at least 5 mM, preferably in a concentration of 30, 40, 50, 60, 70, 80, 90, 100, 150 mM, more preferably in a concentration > 300 mM.
87. Method according to any one of claim 85 or 86, wherein said lactose feed is accomplished by adding lactose to the cultivation in a concentration, such, that throughout the production phase of the cultivation a lactose concentration of at least 5 mM, preferably 10 mM or 30 mM is obtained.
88. Method according to any one of claim 42 to 87, wherein the host cells are cultivated for at least about 60, 80, 100, or about 120 hours or in a continuous manner.
89. Method according to any one of claim 42 to 88, wherein said cell is cultivated in a culture medium comprising a carbon source comprising a monosaccharide, disaccharide, oligosaccharide, polysaccharide, polyol, glycerol, a complex medium including molasses, corn steep liquor, peptone, tryptone or yeast extract; preferably, wherein said carbon source is chosen from the list comprising glucose, glycerol, fructose, sucrose, maltose, lactose, arabinose, malto-oligosaccharides, maltotriose, sorbitol, xylose, rhamnose, galactose, mannose, methanol, ethanol, trehalose, starch, cellulose, hemi-cellulose, molasses, corn-steep liquor, high-fructose syrup, acetate, citrate, lactate and pyruvate.
90. Method according to any one of claim 42 to 89, wherein the culture medium contains at least one precursor selected from the group comprising lactose, galactose, fucose, sialic acid, GlcNAc, GalNAc, lacto-N-biose (LNB), N-acetyllactosamine (LacNAc).
91. Method according to any one of claim 42 to 90, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium before the precursor, preferably lactose, is added to the culture medium in a second phase.

92. Method according to any one of claim 42 to 91, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein only a carbon-based substrate, preferably glucose or sucrose, is added to the culture medium.
- 5 93. Method according to any one of claim 42 to 92, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein a carbon-based substrate, preferably glucose or sucrose, and a precursor, preferably lactose, are added to the culture medium.
- 10 94. Method according to any one of claims 42 to 93, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, two-phase partitioning, reverse osmosis, microfiltration, activated charcoal or carbon treatment, treatment with non-ionic surfactants, enzymatic digestion, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.
- 15 95. Method according to any one of claims 42 to 94 further comprising purification of any one of said mammalian milk oligosaccharides from said cell.
96. Method according to any one of claims 95, wherein said purification comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration, 20 electrophoresis, enzymatic treatment or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, drying, lyophilization, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying or vacuum roller drying.
97. Cell according to any one of claims 1 to 41 or method according to any one of claims 42 to 96, wherein 25 said cell is a bacterium, fungus, yeast, a plant cell, an animal cell, or a protozoan cell,
- preferably said bacterium is an *Escherichia coli* strain, more preferably an *Escherichia coli* strain which is a K-12 strain, even more preferably the *Escherichia coli* K-12 strain is *E. coli* MG1655,
 - preferably said fungus belongs to a genus chosen from the group comprising *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*,
 - 30 - preferably said yeast belongs to a genus chosen from the group comprising *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Komagataella*, *Hansenula*, *Yarrowia*, *Starmerella*, *Kluyveromyces* or *Debaromyces*,
 - preferably said plant cell is an algal cell or is derived from tobacco, alfalfa, rice, tomato, cotton, rapeseed, soy, maize, or corn plant,
 - 35 - preferably said animal cell is derived from non-human mammals, birds, fish, invertebrates, reptiles, amphibians or insects or is a genetically modified cell line derived from human cells

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excluding embryonic stem cells, more preferably said human and non-human mammalian cell is an epithelial cell, an embryonic kidney cell, a fibroblast cell, a COS cell, a Chinese hamster ovary (CHO) cell, a murine myeloma cell, an NIH-3T3 cell, a non-mammary adult stem cell or derivatives thereof, more preferably said insect cell is derived from *Spodoptera frugiperda*, *Bombyx mori*,
5 *Mamestra brassicae*, *Trichoplusia ni* or *Drosophila melanogaster*,

- preferably said protozoan cell is a *Leishmania tarentolae* cell.

98. Cell according to claim 97, or method according to claim 97, wherein said cell is a viable Gram-negative bacterium that comprises a reduced or abolished synthesis of poly-N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides,
10 Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or trehalose compared to a non-modified progenitor.

99. Use of a cell according to any one of claim 1 to 41, 97, 98, or method according to any one of claim 42 to 98 for the production of a mixture of at least three different mammalian milk oligosaccharides.

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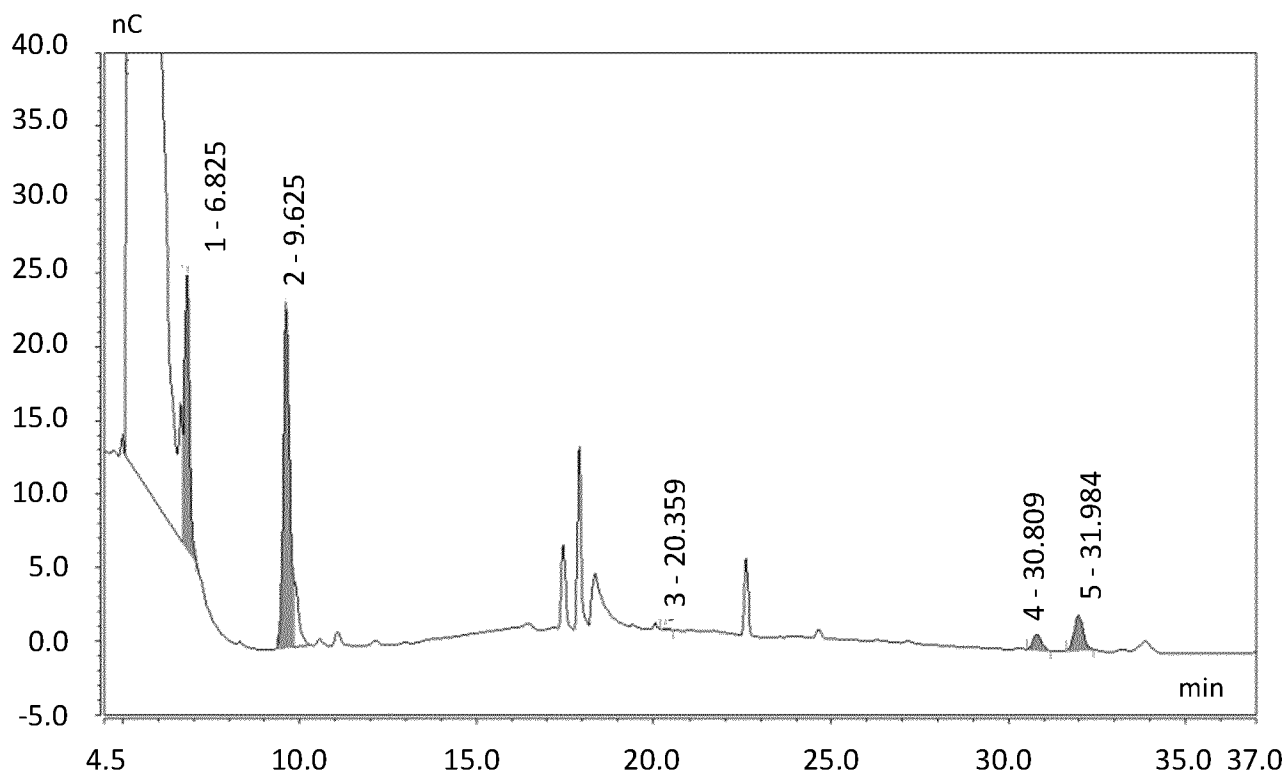


FIGURE 1

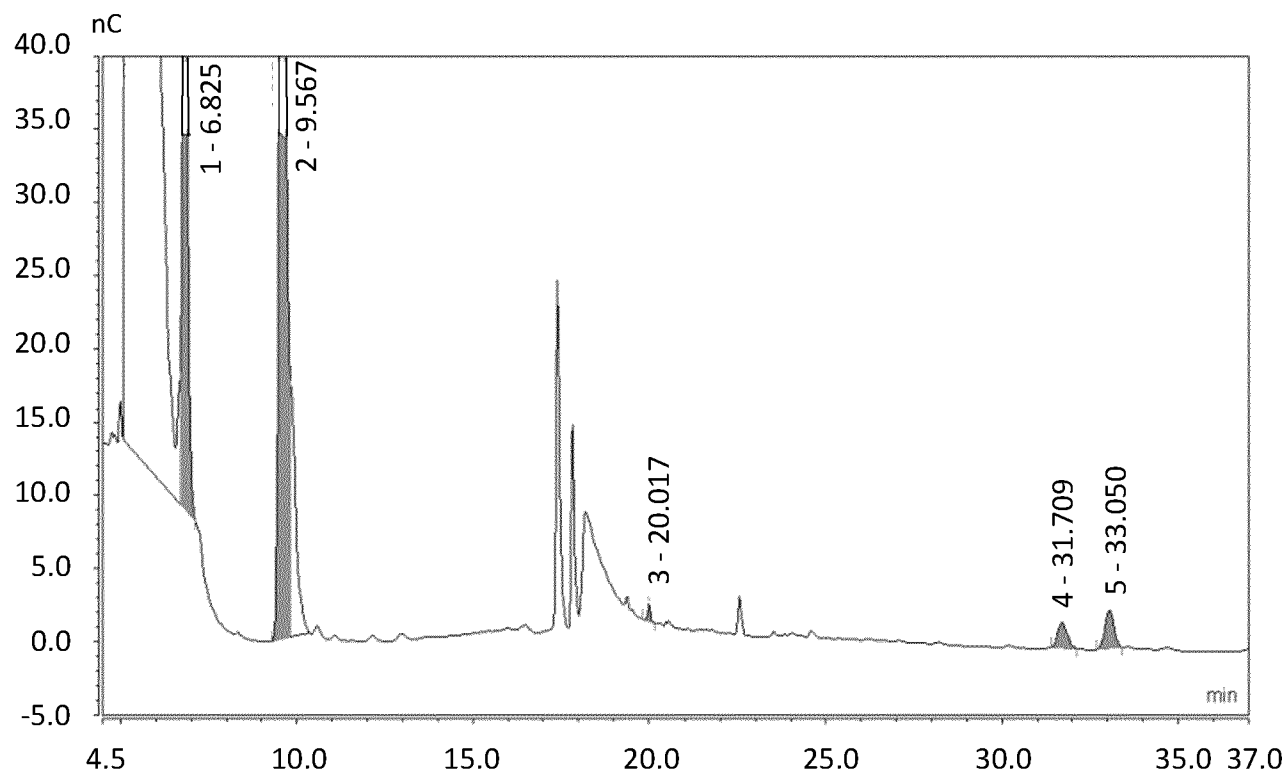


FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/072262

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/04 C12P19/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FAIJES MAGDA ET AL: "Enzymatic and cell factory approaches to the production of human milk oligosaccharides", BIOTECHNOLOGY ADVANCES, ELSEVIER PUBLISHING, BARKING, GB, vol. 37, no. 5, 1 September 2019 (2019-09-01), pages 667-697, XP085733160, ISSN: 0734-9750, DOI: 10.1016/J.BIOTECHADV.2019.03.014 [retrieved on 2019-03-29] paragraph [03.2]; table 11 ----- -/-	1-99



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 October 2021

Date of mailing of the international search report

22/10/2021

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Authorized officer

Ury, Alain

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2021/072262

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOPHIE DROUILLARD ET AL: "Large-Scale Synthesis of H-Antigen Oligosaccharides by Expressing Helicobacter pylori [alpha]1,2-Fucosyltransferase in Metabolically Engineered Escherichia coli Cells", ANGEWANDTE CHEMIE INTERNATIONAL EDITION, vol. 45, no. 11, 6 March 2006 (2006-03-06), pages 1778-1780, XP055105681, ISSN: 1433-7851, DOI: 10.1002/anie.200503427 the whole document -----	1-99
X	DUMON CLAIRE ET AL: "In vivo fucosylation of lacto-N-neotetraose and lacto-N-neohexaose by heterologous expression of Helicobacter pylori alpha-1,3 fucosyltransferase in engineered Escherichia coli", GLYCOCONJUGATE JOURNAL, CHAPMAN & HALL, BOSTON, vol. 18, no. 6, 1 January 2001 (2001-01-01), pages 465-474, XP002380631, ISSN: 0282-0080, DOI: 10.1023/A:1016086118274 the whole document -----	1-99
X	US 2009/082307 A1 (SAMAIN ERIC [FR] ET AL) 26 March 2009 (2009-03-26) the whole document -----	1-99
A	NAM SOO HAN ET AL: "Biotechnological production of human milk oligosaccharides", BIOTECHNOLOGY ADVANCES, vol. 30, no. 6, 1 November 2012 (2012-11-01), pages 1268-1278, XP055074947, ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2011.11.003 the whole document ----- -/--	1-99

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/072262

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WICINSKI MICHAL ET AL: "Human Milk Oligosaccharides: Health Benefits, Potential Applications in Infant Formulas, and Pharmacology", NUTRIENTS , vol. 12, no. 1 20 January 2020 (2020-01-20), page 266, XP055783394, DOI: 10.3390/nu12010266 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7019891/pdf/nutrients-12-00266.pdf the whole document -----	1-99
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/072262

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/072262

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