

Fermentative production of N-acetylneuraminic acid

- 5 The present invention relates to non-naturally-occurring microorganisms that are capable of producing *N*-acetylneuraminic acid, to methods for the production of *N*-acetylneuraminic acid by fermentation using said non-naturally-occurring microorganisms, to the use of *N*-acetylneuraminic acid produced by means of fermentation as well as to products containing *N*-acetylneuraminic acid produced in
10 this manner.

Background

- Sialic acids (Sia) are a family of negatively charged monosaccharides with a nine-carbon backbone. More than 50 forms of these α -keto acids have been found in nature. The most abundant sialic acid appears to be *N*-acetylneuraminic acid
15 (NANA, NeuNAc, Neu5Ac).

- Sialic acids are present as the terminal saccharides of the glycans present in glycoconjugates (glycoproteins and glycolipids) on the surface of cells of vertebrates and higher invertebrates. Sialic acids are components of the lipopolysaccharides and capsular polysaccharides of pathogenic bacteria including *Escherichia coli* K1,
20 *Haemophilus influenzae*, *Haemophilus ducreyi*, *Pasteurella multocida*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campylobacter jejuni* and *Streptococcus agalactiae*.

- Sialic acids play important roles in many physiological and pathophysiological processes including the development of the embryonic nervous system, metastasis, the
25 regulation of immune responses and infections with bacteria or viruses. Sialic acids are an essential component of brain gangliosides and of the polysialic acid chains that modify neural cell adhesion molecules (NCAMs) that facilitate cell-to-cell interactions, neuronal outgrowth, the modification of synaptic connectivity and memory formation. In piglets, a diet rich in sialic acids increases the level of brain

sialic acids and the expression of two learning-related genes. Accordingly, the diet also enhances learning and memory.

Infants, in particular preterm infants, have a high demand for nutrients including sialic acids due to the rapid brain growth and development of their immune system at this developmental stage. Levels of sialic acids, particularly *N*-acetylneuraminic acid, are high in human breast milk (approx. $0.5 \text{ g} \cdot \text{L}^{-1}$). In contrast, infant formulas contain a low or even insignificant amount of *N*-acetylneuraminic acid.

It is therefore necessary to provide sialic acids, in particular Neu5Ac, of a sufficient quality and quantity sufficient for the supplementation of infant formulas and other nutritional compositions. In this regard, various approaches have been published in the past.

Document EP 1 484 406 A1 describes a process for producing *N*-acetylneuraminic acid using a microorganism which has the ability to produce Neu5Ac but has limited or no ability to decompose Neu5Ac compared with a wild-type strain, such that Neu5Ac accumulates in the culture medium and can be recovered therefrom. To enable the production of Neu5Ac, the microorganism possesses strong *N*-acetylneuraminic acid synthase activity and/or *N*-acetylglucosamine 2-epimerase activity. More specifically, *E. coli* cells were subjected to random mutagenesis and a cell line that grew favorably on medium containing glucose but showed limited or no growth on medium containing *N*-acetylneuraminic acid was transformed with an expression plasmid encoding *N*-acetylneuraminic acid synthase and *N*-acetylglucosamine 2-epimerase. After a period of cultivation, the cells were pelleted by centrifugation, stored at $-20 \text{ }^{\circ}\text{C}$ as so-called "wet cells" and used as needed after thawing. For the production of *N*-acetylneuraminic acid, a reaction mixture (30 mL) was provided comprising $90 \text{ g} \cdot \text{L}^{-1}$ *N*-acetylglucosamine, $50 \text{ g} \cdot \text{L}^{-1}$ glucose, $10 \text{ mL} \cdot \text{L}^{-1}$ xylene and $200 \text{ g} \cdot \text{L}^{-1}$ of said wet cells being permeabilized by the presence of $4 \text{ g} \cdot \text{L}^{-1}$ detergent. After completion of the *in-vitro* reaction, the formation of Neu5Ac was evaluated by HPLC.

Document WO 94/29476 A1 discloses an *in vitro* method for the preparation of *N*-acetyl-D-neuraminic acid from *N*-acetyl-D-glucosamine (NAG, GlcNAc). In the preparation, NAG is converted to *N*-acetyl-D-mannosamine (NAM, ManNAc) by base-catalyzed epimerization. Subsequently, NAM reacts with pyruvate in a reaction

catalyzed Neu5Ac-aldolase to yield Neu5Ac. The Neu5Ac-aldolase was prepared from recombinant *E. coli* cells expressing the said Neu5Ac-aldolase. The aldolase enzyme was immobilized by mixing Eupergit-C® beads with a crude extract of said recombinant *E. coli* cells. The conversion of NAM to Neu5Ac was initiated by adding
5 the said immobilized enzyme beads to a mixture of NAM and pyruvate. At the end of the reaction, Neu5Ac was isolated from the reaction mixture.

In an alternative to the previous process, EP 0 578 825 A1 discloses an *in vitro* process for the production of *N*-acetylneuraminic acid by treating a mixture of *N*-acetylglucosamine and pyruvic acid with an *N*-acetylneuraminic acid lyase
10 under alkaline conditions.

US Patent No. US 7,579,175 discloses a process for the production of *N*-acetylneuraminic acid utilizing permeabilized microorganisms. The method comprises the preparation of a mixture containing (i) a culture of a microorganism having *N*-acetylneuraminic acid aldolase activity or *N*-acetylneuraminic acid synthetase activity, or a
15 treated matter of the culture, (ii) a culture of a microorganism capable of producing pyruvic acid or a treated matter of the culture, or a culture of a microorganism capable of producing phosphoenolpyruvic acid or a treated matter of the culture, (iii) *N*-acetylmannosamine, and (iv) an energy source which is necessary for the formation of pyruvic acid or phosphoenolpyruvic acid. The mixture is prepared in an
20 aqueous medium comprising a chelating agent or surfactant allowing the formation and accumulation of *N*-acetylneuraminic acid in the aqueous medium, followed by the recovery of *N*-acetylneuraminic acid from the aqueous medium.

The drawbacks of the aforementioned processes are (i) that only small-scale production is possible and (ii) an excess of pyruvate is required to drive the reaction
25 equilibrium towards Neu5Ac. In addition, *N*-acetylglucosamine, *N*-acetylmannosamine and phosphoenolpyruvate are expensive substrates for these reactions.

International publication WO 2008/040717 A2 discloses a method for the production of sialic acid comprising the cultivation of a microorganism in a medium, wherein said microorganism carries heterologous genes encoding a sialic acid synthase
30 (NeuB) and a UDP-GlcNAc epimerase (NeuC), wherein said microorganism is devoid of a gene encoding CMP-Neu5Ac synthase (NeuA) or wherein any genes

encoding CMP-Neu5Ac synthase (NeuA) have been inactivated or deleted, and wherein endogenous genes coding for sialic acid aldolase (NanA), for the sialic acid transporter (NanT) and, optionally, for ManNAc kinase (NanK) have been deleted or inactivated. Neu5Ac has been purified from the supernatant (2 liters) of a culture by precipitation using glacial acetic acid.

International Publication No. WO 2008/097366 A2 concerns metabolically engineered *E. coli* cells that produce sialic acid. In said cells, the *nanT* (sialic acid transporter) and *nanA* (sialic acid aldolase) genes are inactivated, and the *neuC* and *neuB* genes that facilitate sialic acid biosynthesis in *Neisseria meningitidis* group B are introduced and overexpressed using expression plasmids in said *nanT⁻ nanA⁻* *E. coli* cells. In addition, the *E. coli* glucosamine synthase gene (*glmS*) is co-over-expressed with *neuB* and *neuC*.

International Publication No. WO 2012/083329 A1 discloses methods and agents for the production of Neu5Ac in fungal cells of the genus *Trichoderma*, which constitutively express *N*-acetylglucosamine 2-epimerase and *N*-acetylneuraminic acid synthase. Such *Trichoderma* cells were cultivated in the presence of GlcNAc, and mycelia were analyzed for the presence of Neu5Ac by HPLC-MS.

Chinese Patent Application No. CN 106 929 461 A discloses a process for the production of *N*-acetylneuraminic acid using *Bacillus subtilis* cells which expresses genes encoding for a glucosamine-fructose-6-phosphate transaminase, a glucosamine-6-phosphate *N*-acetyltransferase, a *N*-acetylglucosamine isomerase and a *N*-acetylneuraminic acid synthase. The cells further have the *ptsG* gene deleted, which encodes a glucose-specific component of the phosphotransferase system EIICBA. A yield of 0.66 g·L⁻¹ Neu5Ac was obtained by cultivating these cells in a glucose-containing medium.

Zhu, D. and colleagues (Zhu, D. et al. (2017) *Biotechnol. Lett.* 39: 227-234) report that using a high copy number co-expression vector for overexpression of PEP synthesis-related genes, *pck* and *ppsA* in *E. coli* enhance Neu5Ac production.

It was therefore an objective to provide microbial organisms that are capable of producing sialic acid more efficiently on an industrial scale, and with the use of an inexpensive carbon source as a sole carbon source.

The objective is achieved by providing a non-naturally-occurring microorganism carrying a sialic acid synthesis pathway comprising at least one heterologous enzyme, having the naturally-occurring sialic acid catabolic pathway disabled, being improved with respect to the availability of phosphoenolpyruvic acid for Neu5Ac biosynthesis, and capable of utilizing a single inexpensive exogenous carbon source present in the fermentation broth without using a phosphoenolpyruvic acid:phosphotransferase system for the acquisition of said exogenous carbon source.

Summary

In a first aspect, provided is a non-naturally-occurring microorganism for the production of Neu5Ac, wherein the non-naturally-occurring microorganism possesses a sialic acid synthesis pathway comprising at least one heterologous enzyme, wherein the naturally-occurring sialic acid catabolic pathway has been disabled, wherein at least one phosphotransferase system for the import of a saccharide that is not used as a carbon source during the fermentative production of Neu5Ac has been disabled, and wherein said non-naturally-occurring microorganism can utilize an exogenous carbon source present in the fermentation broth without using a phosphotransferase system for the acquisition of said exogenous carbon source.

In a second aspect, provided is the use of the non-naturally-occurring microorganisms according to the first aspect for the production of Neu5Ac.

In a third aspect, provided is a method for the production of Neu5Ac by fermentation using a non-naturally-occurring microorganism according to the first aspect.

In a fourth aspect, provided is Neu5Ac produced by a method according to the second aspect.

In a fifth aspect, provided is the use of Neu5Ac according to the fourth aspect for the manufacture of a nutritional composition.

In a sixth aspect, provided is a nutritional composition comprising Neu5Ac which has been produced by the method of the third aspect.

Brief description of the drawings

FIG. 1 shows a schematic representation illustrating metabolic pathways for the production of Neu5Ac.

FIG. 2 shows a schematic representation illustrating additional and/or alternative
5 metabolic pathways for the production of Neu5Ac.

FIG. 3 displays a column graph illustrating the levels of Neu5Ac production by different non-naturally-occurring *E. coli* strains.

FIG. 4 displays a column graph illustrating the levels of Neu5Ac production by different non-naturally-occurring *E. coli* strains.

10 Detailed description

According to the first aspect, provided is a non-naturally-occurring microorganism which is able to produce Neu5Ac. Said non-naturally-occurring microorganism possesses a sialic acid biosynthesis pathway comprising at least one heterologous enzyme which is expressed from a heterologous nucleotide sequence in a manner
15 sufficient to produce sialic acid. The naturally-occurring sialic acid catabolic pathway of said microorganisms has been disabled. At least one phosphoenolpyruvate:sugar phosphotransferase system has also been disabled. The non-naturally-occurring microorganism can utilize an exogenously supplied carbon source as a sole carbon source without requiring a phosphoenolpyruvate: sugar phosphotransferase system
20 for the acquisition of said carbon source.

The term “non-naturally-occurring microorganism” as used herein refers to a microorganism that has been genetically engineered to introduce at least one heterologous nucleotide sequence and/or in that a nucleotide sequence which occurs naturally in the microorganism has been modified, i.e. altered, substituted,
25 inserted or deleted.

The term “heterologous” as used herein refers to a compound, a polypeptide, protein, enzyme, nucleic acid molecule or nucleotide sequence - as part of a nucleic

acid molecule - in a host organism which does not naturally have this compound, polypeptide, protein, enzyme, or nucleotide sequence. A "heterologous nucleotide sequence" may be a gene or gene fragment. The term "heterologous expression" refers to the expression of a heterologous gene or gene fragment in a host organism which does not naturally have this gene or gene fragment. Heterologous gene expression leads to the presence of a heterologous polypeptide, protein or enzyme in the host organism.

The non-naturally-occurring microorganism is capable of producing Neu5Ac. The term "producing" as used herein refers to the production of Neu5Ac by microbial fermentation. "Microbial fermentation" is to be understood as a – generally large-scale – industrial process, wherein the desired product, e.g. Neu5Ac, is produced by cultivating a microorganism in a fermentation broth containing nutrients, such that the microorganism can convert compounds to other compounds. The terms "large-scale" and "industrial" indicate that the production can occur by microbial fermentation in a volume of fermentation broth exceeding 100 L, 500 L, 1000 L, 5000 L, 10,000 L 50,000 L 100,000 L or even 200,000 L.

The term "capable of producing" or "able to produce" as used herein refers to the ability of the microorganism to produce Neu5Ac provided that it is cultivated in a medium or broth and under conditions that are permissive for the microorganism to synthesize Neu5Ac.

Sialic acid biosynthesis pathway

The non-naturally-occurring microorganism is a microorganism for the production of Neu5Ac. Therefore, said non-naturally-occurring microorganism is able to produce Neu5Ac. The non-naturally-occurring microorganism is a microorganism that has been genetically engineered to possess a sialic acid biosynthesis pathway.

In an embodiment, the sialic acid biosynthesis pathway of the non-naturally-occurring microorganism comprises at least one heterologous enzyme selected from the group consisting of glutamine–fructose-6-phosphate aminotransferase, glucosamine-6-phosphate *N*-acetyltransferase, *N*-acetylglucosamine 2-epimerase, *N*-acetylneuraminic acid synthase, and a sugar phosphatase of the haloacid

dehydrogenase (HAD)-like superfamily. Preferably, the non-naturally-occurring microorganism is a microorganism that has been genetically engineered to contain one or more of the genes encoding said enzymes. It is to be understood that a host microorganism already carrying one or more genes encoding said enzymes, and
5 expressing said genes in a manner sufficient to produce Neu5Ac, does not need to be genetically engineered to complete sialic acid biosynthesis pathway but may nevertheless be genetically engineered to alter the expression level of one or more of said genes to increase the quantity of glutamine–fructose-6-phosphate amino-transferase, glucosamine-6-phosphate *N*-acetyltransferase, *N*-acetylglucosamine
10 2-epimerase, *N*-acetylneuraminic acid synthase and/or sugar phosphatase of the HAD-like superfamily, thus increasing the rate of Neu5Ac biosynthesis in the non-naturally-occurring microorganism.

The enzyme glutamine–fructose-6-phosphate aminotransferase (EC 2.6.1.16) catalyzes the conversion of fructose 6-phosphate to glucosamine-6-phosphate using
15 glutamine. This enzymatic reaction is typically considered to be the first step in the hexosamine biosynthesis pathway. Alternative names of the glutamine–fructose-6-phosphate aminotransferase are D-fructose-6-phosphate amidotransferase, GFAT, glucosamine-6-phosphate synthase, hexosephosphate aminotransferase, and L-glutamine–D-fructose-6-phosphate amidotransferase.

20 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism possesses a glutamine–fructose-6-phosphate aminotransferase (GlmS), preferably a heterologous glutamine–fructose-6-phosphate amino-transferase, more preferably a glutamine–fructose-6-phosphate aminotransferase which is derived from *E. coli*, or a functional variant of the *E. coli* GlmS. Most
25 preferably, the functional variant is a version of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition as the wild-type enzyme does, for example as encoded by the mutant *glmS* gene (*glmS**54 or *glmS** (see SEQ ID NO: 6).

The term “functional variant” as used herein, with respect to an enzyme, refers to
30 polypeptide variants of the designated enzymes without loss of activity, and which share at least 70%, preferably at least 80 %, more preferably at least 90 % and even more preferably at least 95 % identity with the amino acid sequence of the

designated enzyme. This takes into account the possibility of some variability in the genomic sequence data from which these polypeptides are derived, and also the possibility that some of the amino acids present in these polypeptides can be substituted without significantly affecting the enzyme's catalytic activity.

- 5 The term "functional variants" also includes polypeptide variants of the designated enzymes which represent truncated variants of the enzyme without significant loss of the catalytic activity. Thus, the amino acid sequence of the truncated variants may differ from the amino acid sequences of the designated enzyme in that one, two or a stretch of more than two consecutive amino acids are absent. The truncation may
10 be at the amino terminus (N-terminus), at the carboxyl terminus (C-terminus) and/or within the amino acid sequence of the designated enzyme.

- In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule comprising a nucleotide sequence encoding a glutamine–fructose-6-phosphate aminotransferase. In an additional
15 and/or alternative embodiment, the nucleotide sequence encoding the glutamine–fructose-6-phosphate aminotransferase is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence encoding the glutamine–fructose-6-phosphate aminotransferase encodes the *E. coli* glutamine–fructose-6-phosphate aminotransferase or a functional variant thereof. In an
20 additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to contain a nucleic acid molecule comprising the nucleotide sequence encoding a glutamine–fructose-6-phosphate aminotransferase or functional variant thereof and/or to comprise the glutamine–fructose-6-phosphate aminotransferase or functional variant thereof.

- 25 The *E. coli* glutamine–fructose-6-phosphate aminotransferase (UniProtKB – P17169; SEQ ID NO: 11) is encoded by the *E. coli* *glmS* gene (SEQ ID NO: 10). In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding *E. coli* GlmS or a functional variant thereof,
30 preferably the nucleotide sequence encoding GlmS* (SEQ ID NO: 12 and SEQ ID NO: 13).

In an additional and/or alternative embodiment, the nucleotide sequence encoding the *E. coli* GlmS or one of the functional variants of the *E. coli* GlmS has a sequence identity to *E. coli glmS* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

- 5 The enzyme glucosamine-6-phosphate *N*-acetyltransferase (Gna1, EC 2.3.1.4) converts glucosamine-6-phosphate to *N*-acetylglucosamine-6-phosphate using acetyl-CoA. This enzymatic reaction is considered to be the first step of the subpathway that synthesizes *N*-acetyl- α -D-glucosamine 1-phosphate from α -D-glucosamine 6-phosphate in *Saccharomyces cerevisiae*. Gna1 is also
10 known as phosphoglucosamine acetylase or phosphoglucosamine transacetylase.

- In an additional and/or alternative embodiment, the non-naturally-occurring microorganism comprises a glucosamine-6-phosphate *N*-acetyltransferase (Gna1), preferably a heterologous glucosamine-6-phosphate *N*-acetyltransferase, more preferably a glucosamine-6-phosphate *N*-acetyltransferase which is derived from
15 *S. cerevisiae* (UniProtKB - P43577, SEQ ID NO: 15), or a functional variant of the *S. cerevisiae* Gna1.

- In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule which comprises a nucleotide sequence encoding a glucosamine-6-phosphate *N*-acetyltransferase. In an
20 additional and/or alternative embodiment, the nucleotide sequence encoding the glucosamine-6-phosphate *N*-acetyltransferase is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence encoding the glucosamine-6-phosphate *N*-acetyltransferase encodes the *S. cerevisiae* glucosamine-6-phosphate *N*-acetyltransferase or a functional fragment
25 thereof. However, glucosamine-6-phosphate *N*-acetyltransferases, their deduced amino acid sequences and the nucleotides sequences encoding these glucosamine-6-phosphate *N*-acetyltransferases are known from a variety of different species, and may also be used as suitable glucosamine-6-phosphate *N*-acetyltransferases.
- In an additional and/or alternative embodiment, the non-naturally-occurring
30 microorganism has been genetically engineered to contain a nucleic acid molecule comprising a nucleotide sequence encoding a glucosamine-6-phosphate *N*-acetyl-

transferase or functional variant thereof and/or to comprise a glucosamine-6-phosphate *N*-acetyltransferase or functional variant thereof.

The *S. cerevisiae* glucosamine-6-phosphate *N*-acetyltransferase (UniProtKB - P43577; SEQ ID NO: 15) is encoded by the *S. cerevisiae* *gna1* gene (SEQ ID NO: 14). In an additional and/or alternative embodiment, the non-naturally-occurring microorganism comprises nucleic acid molecule comprising and expressing a nucleotide sequence encoding *S. cerevisiae* Gna1 or a functional variant thereof, preferably the nucleotide sequence encoding *S. cerevisiae* Gna1 (SEQ ID NO: 14).

In an additional and/or alternative embodiment, the nucleotide sequence encoding the *S. cerevisiae* Gna1 or one of the functional variants of the *S. cerevisiae* Gna1 has a sequence identity to *S. cerevisiae* *gna1* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism expresses a sugar phosphatase of the HAD-like superfamily which catalyzes the conversion of *N*-acetylglucosamine-6-phosphate (GlcNAc6P) to *N*-acetylglucosamine (GlcNAc). The HAD-like superfamily is named after the bacterial enzyme haloacid dehydrogenase and includes phosphatases. A suitable phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc may be selected from the group consisting of fructose-1-phosphate phosphatase (YqaB, UniProtKB - P77475) and alpha-D-glucose 1-phosphate phosphatase (YihX, UniProtKB – P0A8Y3). The *E. coli* YqaB and *E. coli* YihX enzymes are considered to also act on GlcNAc6P (Lee, S.-W. and Oh, M.-K. (2015) Metabolic Engineering 28: 143-150). In an embodiment, the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc-6-phosphate to GlcNAc is a heterologous enzyme in the non-naturally-occurring microorganism. In an additional and/or alternative embodiment, the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is selected from the group consisting of *E. coli* YqaB, *E. coli* YihX, and functional variants thereof.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule which comprises a nucleotide sequence encoding a sugar phosphatase of the HAD-like superfamily catalyzing the conver-

sion of GlcNAc6P to GlcNAc. In an additional and/or alternative embodiment, the nucleotide sequence encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence
5 encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc encodes The *E. coli* fructose-1-phosphate phosphatase or the *E. coli* alpha-D-glucose 1-phosphate phosphatase or a functional fragment of one of these two enzymes.

In an additional and/or alternative embodiment, the non-naturally-occurring micro-
10 organism has been genetically engineered to contain a nucleic acid molecule comprising a nucleotide sequence encoding a sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc or a functional fragment of said HAD phosphatase and/or to comprise a sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc or functional
15 variant thereof.

Nucleotide sequences encoding a suitable sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc may be selected from the group of nucleotide sequences encoding *E. coli* YqaB, *E. coli* YihX and functional variants thereof.

20 *E. coli* YqaB (SEQ ID NO: 17) and *E. coli* YihX (SEQ ID NO: 19) are encoded by *E. coli* genes *yqaB* (SEQ ID NO: 16) and *yihX* (SEQ ID NO: 18) respectively. Thus, in an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule comprising a nucleotide sequence encoding *E. coli* YqaB, *E. coli* YihX, or a functional fragment of one of these two
25 enzymes.

In an additional and/or alternative embodiment, the nucleotide sequence encoding *E. coli* YqaB or functional variant thereof has a sequence identity to *E. coli yqaB* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

In an additional and/or alternative embodiment, the nucleotide sequence encoding
30 *E. coli* YihX or functional variant thereof has a sequence identity to *E. coli yihX* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

N-acetylglucosamine 2-epimerase (EC 5.1.3.8) is an enzyme that catalyzes the conversion of *N*-acetylglucosamine (GlcNAc) to *N*-acetylmannosamine (ManNAc). The enzyme is a racemase acting on carbohydrates and their derivatives. The systematic name of this enzyme class is *N*-acyl-D-glucosamine 2-epimerase. This
5 enzyme participates in amino-sugar metabolism and nucleotide-sugar metabolism.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism possesses a *N*-acetylglucosamine 2-epimerase, preferably a heterologous *N*-acetylglucosamine 2-epimerase.

In an additional and/or alternative embodiment, the *N*-acetylglucosamine 2-
10 epimerase is derived from *Anabena variabilis*, *Acaryochloris* sp., *Nostoc* sp., *Nostoc punctiforme*, *Bacteroides ovatus* or *Synechocystis* sp. or is a functional variant thereof. The *N*-acetylglucosamine 2-epimerase of *B. ovatus* ATCC 8483 (UniProtKB - A7LVG6, SEQ ID NO: 21) is encoded by gene BACOVA_01816 (SEQ ID NO: 20). The *Synechocystis* sp. (strain PCC 6803) *N*-acetylglucosamine 2-epimerase
15 (UniProtKB - P74124; SEQ ID NO: 23) is also known as renin-binding protein and is encoded by the *slr1975* gene (SEQ ID NO: 22).

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule comprising a nucleotide sequence encoding the *N*-acetylglucosamine 2-epimerase or functional variant thereof. In an
20 additional and/or alternative embodiment, the nucleotide sequence encoding the *N*-acetylglucosamine 2-epimerase is selected from the group consisting of nucleotide sequences encoding the *N*-acetylglucosamine 2-epimerase of *Anabena variabilis*, *Acaryochloris* sp., *Nostoc* sp., *Nostoc punctiforme*, *Bacteroides ovatus* or *Synechocystis* sp., and functional variants thereof. In an additional and/or alternative
25 embodiment, the nucleotide sequence encoding the *N*-acetylglucosamine 2-epimerase is a heterologous nucleotide sequence.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to contain a nucleic acid molecule comprising the nucleotide sequence encoding an *N*-acetylglucosamine 2-epimerase
30 or functional variant thereof and/or to comprise the *N*-acetylglucosamine 2-epimerase or functional variant thereof.

In an additional and/or alternative embodiment, the nucleotide sequence encoding one of the functional variants of the *N*-acetylglucosamine 2-epimerase has a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *Synechocystis* sp. *slr1975* gene.

- 5 In an additional and/or alternative embodiment, the non-naturally-occurring micro-organism possesses GlcNAc-6-phosphate epimerase activity and ManNAc-6-phosphate phosphatase activity.

- GlcNAc-6-phosphatase epimerase converts GlcNAc-6-phosphate to ManNAc-6-phosphate, whereas ManNAc-6-phosphate phosphatase dephosphorylates
- 10 ManNAc-6-phosphate to give ManNAc. Possessing GlcNAc-6-phosphate epimerase activity and ManNAc-6-phosphate phosphatase activity provides an additional or alternative way in the production of Neu5Ac to convert GlcNAc-6-phosphate to ManNAc as shown in FIG. 2.

- In an additional and/or alternative embodiment, the non-naturally-occurring
- 15 microorganism has been genetically engineered to possess a gene encoding GlcNAc-6-phosphate epimerase or functional variant thereof. Preferably, the non-naturally-occurring microorganism has been genetically engineered to contain a nucleic acid molecule comprising and expressing a nucleotide sequence encoding GlcNAc-6-phosphate epimerase.

- 20 Preferably the GlcNAc-6-phosphate epimerase is derived from *Enterobacter cloacae* subsp. *cloacae* (SEQ ID NO: 25) or a functional variant thereof. The nucleotide sequence encoding GlcNAc-6-phosphate epimerase of *E. cloacae* subsp. *cloacae* is the protein-coding region of the *nanE* gene of *Enterobacter cloacae* subsp. *cloacae* ATCC 13047 (SEQ ID NO: 24).

- 25 In an additional and/or alternative embodiment, the nucleotide sequence encoding one of the functional variants of GlcNAc-6-phosphate epimerase has a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. cloacae* subsp. *cloacae nanE* gene.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to possess a gene encoding ManNAc-6-phosphate phosphatase or a functional variant thereof.

N-acetylneuraminic acid synthase (EC 2.5.1.56) is an enzyme that catalyzes the conversion of *N*-acetylmannosamine (ManNAc) to Neu5Ac using phosphoenolpyruvate (PEP). The *N*-acetylneuraminic acid synthase (NeuB) is encoded by the *neuB* gene.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism comprises an *N*-acetylneuraminic acid synthase or a functional variant thereof, preferably a heterologous *N*-acetylneuraminic acid synthase. In an additional embodiment, the *N*-acetylneuraminic acid synthase is derived from *Campylobacter jejuni* SEQ ID NO: 29), *Streptococcus agalactiae*, *Butyrivibrio proteoclasticus*, *Methanobrevibacter ruminantium*, *Acetobacterium woodii*, *Desulfobacula toluolica*, *Escherichia coli*, *Prevotella nigescens*, *Halorhabdus tiamatea*, *Desulfotignum phosphitoxidans*, or *Candidatus Scalindua* sp., *Idomarina loihiensis*, *Fusobacterium nucleatum* or *Neisseria meningitidis*.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism contains a nucleic acid molecule comprising a nucleotide sequence encoding an *N*-acetylneuraminic acid synthase or a functional variant thereof. In an additional and/or alternative embodiment, the nucleotide sequence encoding the *N*-acetylneuraminic acid synthase is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence encoding the *N*-acetylneuraminic acid synthase is selected from the group consisting of nucleotide sequences encoding *C. jejuni* NeuB (SEQ ID NO: 28), *S. agalactiae* NeuB, *B. proteoclasticus* NeuB, *M. ruminantium* NeuB, *A. woodii* NeuB, *D. toluolica* NeuB, *E. coli* NeuB, *P. nigescens* NeuB, *H. tiamatea* NeuB, *D. phosphitoxidans* NeuB, *Ca. scalindua* sp. NeuB, *I. loihiensis* NeuB, *F. nucleatum* NeuB, *N. meningitidis* NeuB and functional variants thereof.

In an additional and/or alternative embodiment, the nucleotide sequence encoding the *N*-acetylneuraminic acid synthase or one of the functional variants of the *N*-acetylneuraminic acid synthase has a sequence identity of at least 80%, at least

- 85%, at least 90%, at least 95%, at least 98% or at least 99% to one of the nucleotide sequences encoding *C. jejuni* NeuB, *S. agalactiae* NeuB, *B. proteo-*
clasticus NeuB, *M. ruminatum* NeuB, *A. woodii* NeuB, *D. toluolica* NeuB, *E. coli*
 NeuB, *P. nigescens* NeuB, *H. tiamatea* NeuB, *D. phosphitoxidans* NeuB, *Ca.*
 5 *Scalindua* sp. NeuB, *I. loihiensis* NeuB, *F. nucleatum* NeuB, *N. meningitidis* NeuB

Sialic acid catabolic pathway

- The non-naturally-occurring microorganism for the production of Neu5Ac can not utilize Neu5Ac. In an additional embodiment, the non-naturally-occurring microorganism has been genetically engineered such that it does not utilize sialic acid.
- 10 Thereby, the Neu5Ac that has been synthesized by the non-naturally-occurring microorganism is neither degraded in naturally occurring catabolic pathways, nor incorporated into lipopolysaccharides and/or into polysialic acids. Instead, the non-naturally-occurring microorganism is capable of secreting the Neu5Ac it has synthesized into the culture medium or fermentation broth.
- 15 The non-naturally-occurring microorganism is able to produce Neu5Ac. For being able to produce Neu5Ac, the naturally-occurring sialic acid catabolic pathway has been disabled. Disruption of the sialic acid catabolic pathway in the microorganism prevents any sialic acid synthesized by that microorganism from being metabolized further, and thus increases the yield of sialic acid that can be produced by the non-
- 20 naturally-occurring microorganism.

In an additional and/or alternative embodiment, the naturally-occurring sialic acid catabolic pathway is disabled by genetically engineering the microorganism.

- In an additional and/or alternative embodiment, the naturally occurring sialic acid catabolic pathway has been disrupted by deleting or otherwise mutating one or more
- 25 of the genes encoding enzymes required for sialic acid catabolism. The enzyme(s) required for sialic acid catabolism are therefore no longer produced, or are produced at much lower level than normal, e.g. in the wild type microorganism. For example, one or more genes encoding enzymes required for sialic acid catabolism can be deleted from the genome, such that the corresponding enzymes are not produced at
- 30 all. Alternatively, the regulatory sequences controlling gene expression can be

replaced or mutated so that the gene can not be transcribed or translated. This impairment of transcription or translation includes permanent impairment of transcription or translation as well as transient impairment of transcription or translation. I.e. transcription or translation of the respective gene can be regulated
5 by inducing or repressing transcription or translation. Thus, expression of a respective gene can be induced at any desired point of time during cultivation of the microorganism, preferably by adding a compound inducing expression of the respective gene (inducer) to the culture medium. In another embodiment, expression of a respective gene can be repressed at any desired point of time
10 during cultivation of the microorganism, preferably by adding a compound which represses expression of the respective gene (repressor) to the culture medium or by depleting the culture medium of any compound acting as an inducer. In a different approach, the nucleotide sequence encoding an enzyme required for sialic acid catabolism can be altered in such a way that the activity of the enzyme is abolished.
15 This may be achieved by altering the nucleotide sequence to replace a sense codon (specifying an amino acid) in the original nucleotide sequence with a stop codon such that a truncated polypeptide is generated which lacks the activity of the enzyme required for sialic acid catabolism, or by replacing a sense codon with another codon specifying a different amino acid, which produces a non-functional
20 variant of the enzyme required for sialic acid catabolism.

In an additional and/or alternative embodiment, the genes targeted for disruption or alteration to abolish sialic acid catabolism in the non-naturally-occurring microorganism encode one or more enzymes selected from the group consisting of *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase,
25 *N*-acetylneuraminic acid aldolase and sialic acid permease.

N-acetylmannosamine kinase (EC 2.7.1.60) is an enzyme that phosphorylates *N*-acetylmannosamine to yield *N*-acetylmannosamine-6-phosphate. The *N*-acetylmannosamine kinase is encoded by the *nanK* gene. The nucleotide sequence of the protein-coding region of *E. coli nanK* is represented by SEQ ID NO: 30.

30 *N*-acetylmannosamine-6-phosphate epimerase is an enzyme that converts *N*-acetylmannosamine-6-phosphate (ManNAc-6-P) to *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P). This enzymatic reaction is a step of the subpathway that synthesizes

D-fructose 6-phosphate from *N*-acetylneuraminate. The *N*-acetylmannosamine-6-phosphate epimerase is encoded by the *nanE* gene. The nucleotide sequence of the protein-coding region of *E. coli nanE* is represented by SEQ ID NO: 32.

N-acetylneuraminic acid aldolase, also called *N*-acetylneuraminate lyase, catalyzes the reversible aldol cleavage of *N*-acetylneuraminic acid to form pyruvate and *N*-acetylmannosamine (ManNAc). *N*-acetylneuraminic acid aldolase is encoded by the *nanA* gene. The nucleotide sequence of the protein-coding region of *E. coli nanA* is represented by SEQ ID NO: 34.

Sialic acid permease catalyzes the proton-dependent transport of sialic acid across the cell membrane. Sialic acid permease can transport *N*-acetylneuraminic acid. Variants of sialic acid permease can also transport the related sialic acids *N*-glycolylneuraminic acid (Neu5Gc) and 3-keto-3-deoxy-D-glycero-D-galactononic acid (KDN). Although sialic acid permease is known to function as a bidirectional transporter *in vitro*, it accounts for the cellular import of extracellular Neu5Ac *in vivo*. The sialic acid permease is encoded by the *nanT* gene. The nucleotide sequence of the protein-coding region of *E. coli nanT* is represented by SEQ ID NO: 36. Disruption of *nanT* prevents reimport of Neu5Ac that has been produced and secreted into the culture medium by the non-naturally-occurring microorganism.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has reduced or abolished activity of at least one of the enzymes selected from the group consisting of *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase, *N*-acetylneuraminic acid aldolase and sialic acid permease, as compared to the wildtype microorganism. In an additional and/or alternative embodiment, the microorganism has been genetically engineered to have reduced or abolished activity of at least one of these enzymes. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to completely delete one or more of the genes encoding *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase, *N*-acetylneuraminic acid aldolase and sialic acid permease, to impair the expression of one or more of those genes, or to abolish the activity of one or more of the corresponding enzymes by introducing mutations into the protein-coding region of the gene(s) such that the polypeptide encoded by the altered nucleotide sequence

does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism does not possess an enzymatic activity provided by at least one of the enzymes *N*-acetylglucosamine-6-phosphate deacetylase and *N*-acetylglucosamine-6-phosphate deaminase.

In an additional and/or alternative embodiment, at least one of the enzymes *N*-acetylglucosamine-6-phosphate deacetylase and *N*-acetylglucosamine-6-phosphate deaminase has been disabled in the non-naturally-occurring microorganism.

N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25) is the enzyme involved in the first step in the biosynthesis of amino-sugar-nucleotides. It catalyzes the hydrolysis of the *N*-acetyl group of *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) to yield glucosamine 6-phosphate and acetate. The *N*-acetylglucosamine-6-phosphate deacetylase is encoded by the *nagA* gene. The nucleotide sequence of the protein coding region of *E. coli nagA* is represented by SEQ ID NO: 38.

Glucosamine-6-phosphate deaminase (EC 3.5.99.6) catalyzes the reversible isomerization-deamination of glucosamine 6-phosphate (GlcN6P) to form fructose 6-phosphate (Fru6P). Glucosamine-6-phosphate deaminase is encoded by the *nagB* gene. The nucleotide sequence of the protein coding region of *E. coli nagB* is represented by SEQ ID NO: 40.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to disable *N*-acetylglucosamine-6-phosphate deacetylase and/or Glucosamine-6-phosphate deaminase. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to completely delete one or more of the genes encoding *N*-acetylglucosamine-6-phosphate deacetylase and Glucosamine-6-phosphate deaminase, to impair the expression of one or more of those genes, or to abolish the activity of one or more of the corresponding enzymes by introducing mutations into the protein-coding region of at least one of those genes such that the polypeptide

encoded the altered nucleotide sequence does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.

Phosphotransferase carbohydrate transport system

The intracellular production of sialic acid requires phosphoenolpyruvate (PEP). PEP
5 is a very important metabolic intermediate because it is involved in glycolysis and gluconeogenesis. To improve the production of Neu5Ac, the non-naturally-occurring microorganism has been genetically engineered to provide a better supply of PEP for sialic acid biosynthesis. To this end, the non-naturally-occurring microorganism has been genetically engineered in that at least one PEP-dependent, sugar-
10 transporting phosphotransferase system (PTS) has been disabled, i.e. the corresponding gene has been deleted or disrupted, or the expression of the gene has been impaired.

A PEP-dependent, sugar-transporting phosphotransferase system suitable for disruption is GlcNAc permease, also known as protein-Npi-phospho-L-histidine:N-acetyl-D-glucosamine Npi-phosphotransferase (EC 2.7.1.193), which is encoded by
15 the *nagE* gene. NagE (known as enzyme II) is a component of a PEP-dependent, sugar transporting phosphotransferase system. The system simultaneously transports its substrate from the periplasm or extracellular space into the cytoplasm and phosphorylates it. The deletion or disruption of *nagE* or the impairment of its
20 expression is advantageous, because this prevents the import of GlcNAc at the expense of PEP, which would otherwise reduce the amount of PEP available for sialic acid production. Hence, the deletion or disruption of *nagE* or the impairment of its expression increases the intracellular pool of PEP that can be utilized by the non-naturally-occurring microorganism to produce Neu5Ac, thereby increasing the
25 Neu5Ac yield in the non-naturally-occurring microorganism as compared to a non-naturally-occurring microorganism that can produce sialic acid, but which carries an intact and functional *nagE* gene. The nucleotide sequence of the protein coding region of *E. coli nagE* is represented by SEQ ID NO: 42.

In an additional and/or alternative embodiment, the non-naturally-occurring
30 microorganism has been genetically engineered to abolish protein-Npi-phospho-L-histidine:N-acetyl-D-glucosamine Npi-phosphotransferase activity.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to completely delete the *nagE* gene, to impair its expression, or to abolish the activity of the NagE enzyme by introducing mutations into the protein-coding region of the *nagE* gene such that the polypeptide encoded the altered nucleotide sequence does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.

Another or additional PEP-dependent, sugar-transporting phosphotransferase system for import of a carbohydrate that is suitable for disruption is the mannose permease.

ManXYZ, the Enzyme II^{Man} complex (mannose PTS permease, protein-Npi-phosphohistidine-D-mannose phosphotransferase) imports exogenous hexoses (mannose, glucose, glucosamine, fructose, 2-deoxyglucose, mannosamine, N-acetylglucosamine, etc.) and releases the phosphate esters into the cell cytoplasm. This enzyme is also a component of a PEP-dependent, sugar-transporting phosphotransferase system. ManXYZ possesses four domains in three polypeptide chains, ManX=IIAB^{Man}, ManY=IIC^{Man} and ManZ=IID^{Man}. They are members of the mannose PTS permease family, the splinter group, which is not homologous to most other PTS permeases. The nucleotide sequences of the protein-coding regions of *E. coli manX*, *manY* and *manZ* are represented by SEQ ID NO: 44, SEQ ID NO: 46 and SEQ ID NO: 48 respectively.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to abolish protein-Npi-phospho-L-histidine:mannose Npi-phosphotransferase activity.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to completely delete one or more of the genes encoding ManX, ManY and ManZ, to impair the expression of one or more of those genes, or to abolish the activity of one or more of the corresponding enzymes by introducing mutations by introducing mutations into the protein-coding region(s) of the gene(s) such that the polypeptide encoded the altered nucleotide

sequence does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.

Another or additional PEP-dependent, sugar-transporting phosphotransferase system suitable for disruption is the glucose transporter.

- 5 The glucose-specific PTS transporter (PtsG/Crr) takes up exogenous glucose, releasing the phosphate ester into the cytoplasm. The enzyme IIC^{Glc} complex possesses two domains in a single polypeptide chain with the domain order IIC-IIB (PtsG), and it functions with an additional polypeptide chain, the Crr or IIA^{Glc} protein.

- 10 The deletion or disruption of *ptsG* and/or *crr* or the impairment of its expression is advantageous, because this prevents the import of glucose at the expense of PEP, which would otherwise reduce the amount of PEP available for sialic acid production. Hence, the deletion or disruption of the *ptsG* gene and/or the *crr* gene or impairment of its expression increases the intracellular pool of PEP that can be utilized by the non-naturally-occurring microorganism to produce Neu5Ac, thereby
15 increasing the Neu5Ac yield in the non-naturally-occurring microorganism that can produce Neu5Ac as compared to a non-naturally occurring microorganism that can produce sialic acid, but which carries an intact and functional *ptsG* and/or *crr* gene.

The nucleotide sequences of the protein-coding regions of *E. coli ptsG* and *crr* are represented by SEQ ID NO: 50 and SEQ ID NO: 52 respectively.

- 20 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to abolish PtsG/Crr activity.

- 25 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to completely delete the *ptsG* gene and/or the *crr* gene, to impair the expression of the *ptsG* gene and/or the *crr*, or to abolish the activity of PtsG/Crr by introducing mutations into the protein-coding region of the *ptsG* gene and/or the *crr* gene such that the polypeptide encoded by the altered nucleotide sequence(s) does not possess the enzymatic activity of the enzyme(s) encoded by the non-amended nucleotide sequence.

Acquisition of carbon source

The non-naturally-occurring microorganism requires a carbon source for growth, proliferation and production of Neu5Ac. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism may grow on an inexpensive sole carbon source, such as – for example – glucose or sucrose. Said
5 sole carbon source provides an educt for sialic acid biosynthesis in the non-naturally-occurring microorganism. Hence, for the production of Neu5Ac it is not necessary to cultivate the non-naturally-occurring microorganism in the presence of ManNAc, GlcNAc or glucosamine (GlcN). In addition, the non-naturally-occurring microorganism does not require a PEP-dependent, sugar-transporting
10 phosphotransferase system for import of the sole carbon source, and hence does not need to utilize PEP for the acquisition of the sole carbon source.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to utilize sucrose as the sole carbon source.

15 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism possesses a functional sucrose utilization system. Said functional sucrose utilization system enables cellular import of exogenously supplied sucrose and its hydrolysis such that the resulting monosaccharides glucose and fructose can be metabolically utilized by the non-naturally-occurring microorganism's metabolism
20 and for the desired Neu5Ac production.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically modified to possess a functional sucrose utilization system. In an additional and/or alternative embodiment the sucrose utilization system of the non-naturally-occurring microorganism comprises a sucrose
25 proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor.

A suitable a sucrose proton symport transport system is CscB, encoded by the *cscB* gene, for example CscB of *E. coli* (SEQ ID NO: 55) as encoded by the *cscB* gene of *E. coli* (SEQ ID NO: 54).

A suitable fructokinase (EC 2.7.1.4) is CscK, encoded by the *cscK* gene, for example CscK of *E. coli* (SEQ ID NO: 57) as encoded by the *cscK* gene of *E. coli* (SEQ ID NO: 56).

- 5 A suitable invertase (EC 3.2.1.26) which hydrolysis terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides is CscA, for example *cscA* of *E. coli* (SEQ ID NO: 59) as encoded by the *cscA* gene of *E. coli* (SEQ ID NO: 58).

A suitable sucrose operon repressor is CscR as encoded by the *cscR* gene, for example the CscR of *E. coli* (SEQ ID NO: 61) as encoded by the *cscR* gene of *E. coli* (SEQ ID NO: 60).

- 10 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to possess a sucrose proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor or functional variants of any one of these proteins.

- 15 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to possess a nucleic acid molecule comprising nucleotide sequences encoding a sucrose proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor for the expression of said sucrose proton symport transport system, fructokinase, invertase and sucrose operon repressor. In an additional and/or alternative embodiment, the
20 non-naturally-occurring microorganism has been genetically engineered to express the genes *cscB*, *cscK*, *cscA*, preferably the *E. coli* genes *cscB*, *cscK*, *cscA* and *cscR*.

- In an additional and/or alternative embodiment, the nucleotide sequence encoding a functional variant of CscB, CscK, CscA or CscR has a sequence identity or at least
25 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to *E. coli* *cscB*, *cscK*, *cscA* or *cscR*, respectively.

- The non-naturally-occurring microorganism that can produce Neu5Ac and carries a functional sucrose utilization system can be cultivated in the presence of sucrose as a sole carbon source for the microorganism's metabolism as well as Neu5Ac
30 biosynthesis. Sucrose is an inexpensive sugar and its utilization as sole carbon

source for the production of Neu5Ac by fermentation is more cost efficient than other sialic acid precursors, such as GlcNAc.

Another suitable saccharide utilization system allows the non-naturally-occurring microorganism to grow on a sole carbon source without a PEP-dependent, sugar-transporting phosphotransferase system is LacY, encoded by the *lacY* gene of the *lac* operon. LacY is a β -galactoside permease which imports lactose across cell membranes using a proton gradient in the same direction. Intracellular lactose may be hydrolyzed by a β -galactosidase (LacZ) to provide glucose and galactose within the cell. The *lacZ* gene is also part of the *lac* operon.

- 10 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism expresses a β -galactoside permease and a β -galactosidase.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to express β -galactoside permease, preferably the *E. coli* lactose permease LacY (SEQ ID NO: 63) or a functional variant thereof and β -galactosidase, preferably *E. coli* LacZ (SEQ ID NO: 65) or a functional variant thereof. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to carry a nucleic acid molecule comprising a nucleotide sequence encoding a β -galactoside permease, preferably a nucleotide sequence encoding the *E. coli* LacY (SEQ ID NO: 62) or a functional variant thereof, and/or a nucleotide sequence encoding a β -galactosidase, preferably a nucleotide sequence encoding *E. coli* LacZ (SEQ ID NO: 64) or a functional variant thereof.

In an additional and/or alternative embodiment, the nucleotide sequence encoding *E. coli* LacY or a functional variant thereof has a sequence identity to *E. coli lacY* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

In an additional and/or alternative embodiment, the nucleotide sequence encoding *E. coli* LacZ or a functional variant thereof has a sequence identity to *E. coli lacZ* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

A non-naturally-occurring microorganism that can produce Neu5Ac, and which expresses a functional β -galactoside permease and a functional β -galactosidase

allows the cultivation of said non-naturally-occurring microorganism on lactose as a sole carbon source.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism expresses a glucose/H⁺-symporter. Preferably, the non-naturally-occurring microorganism has been genetically engineered to carry a nucleic acid molecule comprising a nucleotide sequence encoding and allowing the expression of a glucose/H⁺-symporter in said non-naturally-occurring microorganism.

A suitable glucose/H⁺-symporter is selected from the group consisting of the *Staphylococcus epidermis* glucose/H⁺-symporter (UniProtKB – A0A0U5QDM9; SEQ ID NO: 67), the *Lactobacillus brevis* glucose/H⁺-symporter (UniProtKB – A0A0C1PU75, SEQ ID NO: 69) and functional variants thereof.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to carry a nucleic acid molecule encoding the *S. epidermis* glucose/H⁺-symporter or the *L. brevis* glucose/H⁺-symporter. Preferably, the non-naturally-occurring microorganism carries a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 66, SEQ ID NO: 68, and nucleotide sequences having a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to SEQ ID NO: 66 or SEQ ID NO: 68.

The non-naturally occurring microorganism that can produce Neu5Ac, and that expresses either the *S. epidermis* glucose/H⁺-symporter or the *L. brevis* glucose/H⁺-symporter, can be cultivated in the presence of glucose as a sole carbon source without needing PEP for the acquisition of the exogenously supplied glucose.

Additional genetic modifications

The non-naturally-occurring microorganism that can produce Neu5Ac may – optionally – include additional features, and may be genetically engineered to possess these additional features. These additional features are considered to improve the productivity of the non-naturally-occurring microorganism leading to higher Neu5Ac yields.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism synthesizes more PEP than the wildtype of the microorganism. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to possess an enhanced PEP biosynthesis pathway. Preferably, the non-naturally-occurring microorganism has been 5 genetically engineered to possess an increased phosphoenolpyruvate synthase activity, for example in that the *ppsA* gene encoding phosphoenolpyruvate synthase gene is overexpressed and/or in that the non-naturally-occurring microorganism contains at least one additional copy of a nucleotide sequence allowing the 10 expression of a phosphoenolpyruvate synthase or a functional variant thereof. Overexpression of *ppsA* enhances intracellular PEP synthesis such that more PEP is available for the production of sialic acid. For example, a suitable phosphoenolpyruvate synthase is PpsA of *E. coli* (SEQ ID NO: 71).

In an additional and/or alternative embodiment, the non-naturally occurring 15 microorganism contains a nucleic acid molecule comprising a nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof. Said nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof has a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. coli ppsA* gene (SEQ ID NO: 70).

20 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to overexpress phosphoenolpyruvate carboxykinase. A suitable phosphoenolpyruvate carboxykinase is *E. coli* Pck (SEQ ID NO: 73).

The enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49) is encoded by the 25 *pck* gene and catalyzes the following reaction: oxaloacetate + ATP → phosphoenolpyruvate + ADP + CO₂. Phosphoenolpyruvate carboxykinase is involved in gluconeogenesis.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to overexpress a phosphoenol- 30 pyruvate carboxykinase and/or to contain at least one additional nucleotide sequence allowing the expression of a phosphoenolpyruvate carboxykinase or a

functional variant thereof. The overexpression of a phosphoenolpyruvate carboxykinase increases the intracellular level of PEP such that more PEP is available for the production of sialic acid.

5 The nucleotide sequence encoding the additional nucleotide sequence encoding phosphoenolpyruvate kinase or a functional variant thereof may be SEQ ID NO: 72 or a nucleotide sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. coli pck* gene (SEQ ID NO: 72).

10 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism does not possess a functional phosphoenolpyruvate carboxylase (EC 4.1.1.31). The phosphoenolpyruvate carboxylase forms oxaloacetate, a four-carbon dicarboxylic acid source for the tricarboxylic acid cycle. The phosphoenolpyruvate carboxylase is encoded by the *ppc* gene. In *E. coli* the phosphoenolpyruvate carboxylase (SEQ ID NO: 27) is encoded by the *pepC* gene (SEQ ID NO: 15 26).

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to abolish PEP carboxylase activity.

20 In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to delete the *ppc* gene or the *pepC* gene, to impair its expression, or to abolish PEP carboxylase activity by introducing mutations into the protein-coding region of the *ppc/pepC* gene such that the polypeptide encoded by the altered nucleotide sequence does not possess PEP carboxylase activity.

25 In an additional and/or alternative embodiment, the non-naturally occurring microorganism has been genetically engineered to have pyruvate kinase activity diminished or abolished.

The enzyme pyruvate kinase generates adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and PEP. The generation of ATP from ADP and PEP is the last step in glycolysis, a step that is irreversible under physiological conditions. 30 Many Enterobacteriaceae, including *E. coli*, have two isoforms of pyruvate kinase,

PykA (SEQ ID NO: 75) and PykF (SEQ IC NO: 77), which are 37% identical in *E. coli*.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to delete one or more genes
5 encoding pyruvate kinase, preferably the *pykA* gene (SEQ ID NO: 74) and/or the *pykF* gene (SEQ ID NO: 76), to impair expression of one or more of those genes encoding pyruvate kinase, or to abolish the activity of at least one pyruvate kinase by introducing one or more mutations into the nucleotide sequence of the protein-
10 coding region of one or more of those genes encoding pyruvate kinase such that the polypeptid encoded by the altered nucleotide sequence does not possess pyruvate kinase activity.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism synthesizes more glutamine as compared to the wild-type. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism
15 has been genetically engineered to possess an enhanced glutamine biosynthesis pathway.

Glutamine synthetase (GlnA) converts glutamate to glutamine by the following reaction: $\text{ATP} + \text{L-glutamate} + \text{NH}_3 = \text{ADP} + \text{phosphate} + \text{L-glutamine}$. In *E. coli*, glutamine synthetase (SEQ ID NO: 79) is encoded by the *glnA* gene (SEQ ID NO:
20 78).

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to overexpress a glutamine synthase and/or to contain at least one additional nucleotide sequence allowing the expression of a glutamine synthase or a functional variant thereof. The
25 overexpression of a glutamine synthase increases the intracellular level of glutamine, which in turn improves the intracellular conversion of fructose-6-phosphate (Frc-6P) to glucosamine-6-phosphate (GlcN-6P). Preferably, the nucleotide sequence encoding the additional nucleotide sequence encoding a glutamine synthase or functional variant thereof may be SEQ ID NO: 78 or a
30 nucleotide sequence having a sequence identity of at least 80%, at least 85%, at

least 90%, at least 95%, at least 98% or at least 99% to the *E. coli glnA* gene (SEQ ID NO: 78).

Metabolic modelling in *E. coli* has confirmed that enhancing glutamine synthesis boosts the production of Neu5Ac. In addition, transcriptome analysis in a Neu5Ac-
5 producing *E. coli* strain (# NANA1) which was not genetically engineered to enhance glutamine synthesis revealed that glutamine synthase was expressed at a higher level compared to a related *E. coli* strain that is not able to produce Neu5Ac.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism does not carry a functional glutamate synthase or has less glutamate
10 synthase activity as compared to a wild-type microorganism. The *E. coli* glutamate synthase consists of two subunits, GltB (SEQ ID NO: 81) and GltD (SEQ ID NO: 83), and synthesizes glutamate at the expense of glutamine. GltB is encoded by the *gltB* gene (SEQ ID NO: 80), and GltD is encoded by the *gltD* gene (SEQ ID NO: 82).

In an additional and/or alternative embodiment, the non-naturally-occurring
15 microorganism has been genetically engineered to delete the *gltB* gene and/or the *gltD* gene, to impair the expression of at least one of those genes, or to reduce or abolish glutamate synthase activity by introducing one or more mutations into the protein-coding region of the *gltB* gene and/or the *gltD* gene such that the polypeptide(s) encoded by the altered nucleotide sequence(s) provide a non-
20 functional variant of the glutamate synthase.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism does not possess glutaminase activity.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to delete at least one of the
25 glutaminase genes *asnB*, *ybaS* and *yneH*, to impair expression of at least one of those genes, or to abolish glutaminase activity by introducing one or more mutations into the protein-coding region of *asnB*, *ybaS* and/or *yneH* such that the polypeptide encoded by one of the altered nucleotide sequences does not possess glutaminase activity.

AsnB is an asparagine synthetase and catalyzes the ATP-dependent conversion of aspartate into asparagine, using glutamine. The *E. coli* asparagine synthetase AsnB (SEQ ID NO: 85) is encoded by the *E. coli asnB* gene (SEQ ID NO: 84).

YbaS, also known as GlcA1 or Glc1, is glutaminase 1, a glutaminase that is highly selective for L-glutamine. YbaS converts L-glutamine to L-glutamate. The *E. coli* glutaminase YbaS (SEQ ID NO: 87) is encoded by the *E. coli ybaS* gene (SEQ ID NO: 86).

YneH, also known as GlcA2, GlcB or glutaminase 2, catalyzes the following reaction: L-glutamine + H₂O = L-glutamate + NH₃. The *E. coli* glutaminase YneH (SEQ ID NO: 89) is encoded by the *E. coli yneH* gene (SEQ ID NO: 88).

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism possesses an increased glutamate dehydrogenase activity as compared to the wild-type microorganism. In an additional and/or alternative embodiment, the non-naturally occurring microorganism has been genetically engineered to overexpress a glutamate dehydrogenase and/or contains at least one additional nucleotide sequence allowing the expression of a glutamate dehydrogenase or a functional variant thereof.

Glutamate dehydrogenase **converts glutamate to α-ketoglutarate**. The overexpression of glutamate dehydrogenase increases the formation of α-ketoglutarate, which in turn can be converted to glutamate by glutamate synthase, for example by glutamate synthase as encoded by *E. coli gltD* or a functional variant thereof. Glutamate can then be converted to glutamine by glutamine synthetase (GlnA) or a functional variant thereof.

In an additional and/or alternative embodiment, the additional nucleotide sequence allowing expression of a glutamate dehydrogenase or functional variant thereof includes the protein-coding region of the *E. coli* glutamate dehydrogenase GdhA (SEQ ID NO: 91). The nucleotide sequence encoding the glutamate dehydrogenase or functional variant thereof may be SEQ ID NO: 90 or a nucleotide sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. coli gdhA* gene (SEQ ID NO: 90).

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism unable to synthesize lipopolysaccharides (LPS) and/or colanic acid.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to abolish the synthesis of LPS
5 and/or colonic acid.

In an embodiment, the non-naturally-occurring microorganism has been genetically engineered to delete the *wzx*C gene, to impair expression of the *wzx*C gene, or to abolish the activity of the WzxC enzyme by introducing one or more mutations into the protein-coding region of the gene the polypeptide being encoded by said altered
10 nucleotide sequence does not possess the enzymatic activity of WzxC. WzxC is required for LPS biosynthesis and encodes a putative export protein. The nucleotide sequence of *E. coli wzx*C is represented by SEQ ID NO: 92, and the deduced amino acid sequence by SEQ ID NO: 93.

In an additional and/or alternative embodiment the non-naturally-occurring micro-
15 organism does not possess UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase activity.

In an additional and/or alternative embodiment the non-naturally-occurring microorganism has been genetically engineered to abolish UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase activity, preferably by deleting
20 the *wca*J gene or a functional variant thereof, by impairing expression of the *wca*J gene or a functional variant thereof, or by abolishing the activity of the WcaJ enzyme by introducing mutations into the protein-coding region of the such that the polypeptide encoded by the altered nucleotide sequence does not possess enzymatic activity of WcaJ. WcaJ encodes a UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase. Said UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase is the first enzyme in colanic acid biosynthesis. The
25 nucleotide sequence of *E. coli wca*J is represented by SEQ ID NO: 94, and the deduced amino acid sequence by SEQ ID NO: 95.

In an additional and/or alternative embodiment, the non-naturally-occurring micro-
30 organism may not comprise a functional β -galactoside permease (LacY) and/or a functional β -galactosidase (LacZ) provided that the non-naturally-occurring

microorganism can be cultivated on another sole carbon source than lactose, for example on sucrose or glucose as sole carbon source.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered in that the β -galactoside permease gene (*lacY*) and/or the β -galactosidase gene (*lacZ*) has been deleted, in that the expression of the β -galactoside permease gene and/or the β -galactosidase gene is impaired or in that the nucleotide sequence of the protein coding region of the β -galactoside permease gene and/or the β -galactosidase gene is amended such that the polypeptide being encoded by said altered nucleotide sequence(s) does not possess the enzymatic activity of the β -galactoside permease and/or the β -galactosidase.

In an additional and/or alternative embodiment, the non-naturally-occurring microorganism does not possess a functional YjhC. YjhC is an oxidoreductase encoded by the *yjhC* gene or a functional variant thereof.

In an additional and/or alternative embodiment the non-naturally-occurring microorganism has been genetically engineered to abolish YjhC oxidoreductase activity, preferably by deleting the *yjhC* gene, by impairing expression of the *yjhC* gene, or by introducing one or more mutations into the protein-coding region of the *yjhC* gene such that the polypeptide being encoded by the altered nucleotide sequence does not possess YjhC oxidoreductase activity.

The nucleotide sequence of *E. coli yjhC* is represented by SEQ ID NO: 96, and the deduced amino acid sequence by SEQ ID NO: 97.

In an additional and/or alternative embodiment, then non-naturally-occurring microorganism does not possess one or more of the following enzyme activities: fucose isomerase, fuculokinase and *N*-acetylglutamine aminoacylase. In an embodiment, the non-naturally-occurring microorganism has been genetically engineered to abolish the activity of one or more of these enzyme activities.

Fucose isomerase converts aldose L-fucose into the corresponding ketose L-fuculose. Fucose isomerase is the first enzyme in the subpathway that synthesizes L-lactaldehyde and glycerone phosphate from L-fucose. The *E. coli* fucose

isomerase FucI (SEQ ID NO: 99) is encoded by the *E. coli fucI* gene (SEQ ID NO: 98).

In an additional and/or alternative embodiment the non-naturally-occurring microorganism has been genetically engineered to abolish fucose isomerase activity, preferably by the deletion the *fucI* gene, by impairing expression of the *fucI* gene, or by modifying the protein-coding region of the *fucI* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess fucose isomerase activity.

Fuculokinase catalyzes the phosphorylation of fucose. Fuculokinase is the second enzyme in the subpathway that synthesizes L-lactaldehyde and glycerone phosphate from L-fucose. The *E. coli* fuculokinase FucK (SEQ ID NO: 101) is encoded by the *E. coli fucK* gene (SEQ ID NO: 100). *E. coli* fuculokinase can also phosphorylate, with lower efficiency, D-ribulose, D-xylulose and D-fructose.

In an additional and/or alternative embodiment the non-naturally-occurring microorganism has been genetically engineered to abolish fucose isomerase activity, preferably by the deletion of the *fucK* gene or, by impairing expression of the *fucK* gene, or by introducing mutations into the protein-coding region of the *fucK* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess fucose isomerase activity.

N-acetylgalactosamine-6-phosphate deacetylase catalyzes the following reaction: $N\text{-acetyl-D-galactosamine 6-phosphate} + H_2O \rightarrow D\text{-galactosamine 6-phosphate} + \text{acetate}$. *N*-acetylgalactosamine-6-phosphate deacetylase is encoded by the *agaA* gene. In contrast to *E. coli* strains C and EC3132, K-12 strains cannot grow on *N*-acetylgalactosamine and D-galactosamine, because they carry a deletion and thus lack an active PTS systems specific for these compounds. Therefore, in K-12 strains, AgaA is not involved in the degradation of these compounds. The *E. coli* AgaA (SEQ ID NO: 103) is encoded by the *E. coli agaA* gene (SEQ ID NO: 102).

In an additional and/or alternative embodiment the non-naturally-occurring microorganism has been genetically engineered to abolish *N*-acetylgalactosamine-6-phosphate deacetylase activity, preferably by deletion of the *agaA* gene, by impairing expression of the *agaA* gene, or by introducing mutations into the protein-

coding region of the *agaA* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess *N*-acetylgalactosamine-6-phosphate deacetylase activity.

The non-naturally-occurring microorganism is selected from the group consisting of yeasts, fungi and bacteria. Preferably, the non-naturally-occurring microorganism is an organism that is generally recognized as safe (GRAS), for example as affirmed by the Federal Drug Administration (FDA) or determined independently by qualified experts, more preferably a prokaryotic microorganism, most preferably a bacterial microorganism. Bacteria suitable for the production of Neu5Ac may be selected from the following genera: *Bacillus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium*, *Sporolactobacillus*, *Micromonospora*, *Micrococcus*, *Rhodococcus*, and *Pseudomonas*. Suitable bacterial species include *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus thermophilus*, *Bacillus laterosporus*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus lentus*, *Bacillus cereus*, *Bacillus circulans*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Citrobacter freundii*, *Clostridium cellulolyticum*, *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*, *Enterococcus faecium*, *Enterococcus thermophiles*, *Escherichia coli*, *Erwinia herbicola* (*Pantoea agglomerans*), *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus jensenii*, *Lactococcus lactis*, *Pantoea citrea*, *Pectobacterium carotovorum*, *Propionibacterium freudenreichii*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Streptococcus thermophiles* and *Xanthomonas campestris*.

According to the second aspect, provided is the use of a non-naturally-occurring microorganism as described herein before for the production of Neu5Ac. The non-naturally-occurring microorganism is capable of producing Neu5Ac in an industrial scale. The terms "**capable**" and "**able**" with respect to the production of Neu5Ac as used herein refers to the ability of the non-naturally-occurring microorganism to synthesize Neu5Ac and to secrete said Neu5Ac into the fermentation broth,

provided that the said non-naturally-occurring microorganism is cultivated under conditions permissive for the production of Neu5Ac. This includes the ability of the said non-naturally-occurring microorganism to proliferate to high cell densities and to be cultured in large volumes, e.g. volumes exceeding 1,000 L, preferably 10,000 L,
5 more preferably 80,000 L and most preferably 200,000 L.

According to the third aspect, provided is a method for the production of Neu5Ac by microbial fermentation. The method comprises the steps of

- providing a non-naturally-occurring microorganism that is able to produce Neu5Ac, preferably a non-naturally-occurring microorganism as described
10 herein before;
- cultivating the said non-naturally-occurring microorganism in a fermentation broth under permissive conditions for the production of Neu5Ac by said microorganism; and - optionally -
- recovering Neu5Ac from the fermentation broth.

15 The fermentation broth contains at least one carbon source for the non-naturally-occurring microorganism. This carbon source is preferably selected from the group consisting of glucose, xylose, fructose, sucrose, lactose, glycerol, syngas and combinations thereof.

In an additional and/or alternative embodiment, the non-naturally-occurring
20 microorganism is cultivated in the absence of and/or without the addition of one or more selected from the group consisting of pyruvate, glucosamine, *N*-acetylglucosamine in the fermentation broth.

The method includes an optional step of recovering Neu5Ac produced by the non-naturally-occurring microorganism during its cultivation in the fermentation broth.

25 The Neu5Ac can be recovered from the fermentation broth after the non-naturally-occurring microorganisms have been removed from the fermentation broth, for example by centrifugation. Subsequently, the Neu5Ac can be further purified from the thus clarified fermentation broth by suitable techniques such as microfiltration, ultrafiltration, diafiltration, simulated moving bed type chromatography,
30 electrodialysis, reverse osmosis, gel filtration, anion exchange chromatography, cation exchange chromatography, and the like.

The method is suitable for the large-scale and economically sustainable production of Neu5Ac by microbial fermentation.

According to the fourth aspect, provided is Neu5Ac, produced by microbial fermentation as described herein.

- 5 According to the fifth aspect, provided is the use of Neu5Ac produced as described herein for the manufacture of a nutritional composition.

According to the sixth aspect, provided is a nutritional composition containing Neu5Ac which has been produced by the method of the third aspect.

- 10 In an additional and/or alternative embodiment, the nutritional composition further contains at least one human milk oligosaccharide (HMO), preferably at least one neutral HMO and/or at least one acidic HMO.

The neutral HMO(s) may be selected from the group consisting of 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT) and lacto-*N*-fucopentaose I (LNPFI).

- 15 The one acidic HMO(s) may be selected from the group consisting of sialylated HMOs, preferably from the group consisting of 3'-sialyllactose (3-SL), 6'-sialyllactose (6-SL), sialyllacto-*N*-tetraose a (LST-a), sialyllacto-*N*-tetraose b (LST-b), sialyllacto-*N*-tetraose c (LST-c) and disialyllacto-*N*-tetraose (DSLNT).

- 20 In an additional embodiment, the nutritional composition is selected from the group consisting of medicinal formulations, infant formula and dietary supplements.

The nutritional composition may be present in liquid form or in solid form including, but not limited to, powders, granules, flakes and pellets.

- 25 In an additional and/or alternative embodiment, the nutritional composition also includes microorganisms, preferably probiotic microorganisms. For infant food applications, preferred microorganisms are derived from or can be found in the microbiome of a healthy human. Preferably, the microorganisms are selected from the genera *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Leuconostoc*, *Clostridium*, *Eubacterium*,

Veillonella, *Fusobacterium*, *Bacterioides*, *Prevotella*, *Escherichia*, *Propionibacterium* or *Saccharomyces*, but others may also be appropriate. In an additional and/or alternative embodiment, the microorganism is selected from the group consisting of *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium lactis*, *Bifido-*
5 *bacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifido-*
bacterium adolescentis, *Lactobacillus acidophilus*, *Lactobacillus plantarum*,
Lactobacillus salivarius, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus*
reuteri, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus salivarius*,
Lactococcus lactis, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, *Lactobacillus*
10 *helveticus*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides*; *Escherichia coli*,
Enterococcus faecium, and *Streptococcus thermophilus* (VSL#3).

In addition to the combination of Neu5Ac with living organisms, Neu5Ac can be also be used in combination with killed cultures which are sometimes used in the field of probiotics (e.g. tyndalized bacteria). Such killed cultures may provide proteins,
15 peptides, oligosaccharides, cell wall fragments, and natural products that cause a short term stimulation of the immune system.

The combination of Neu5Ac and probiotic microorganisms in the nutritional composition is particularly advantageous to establish or re-establish an appropriate microbiome in the gut, and the health benefits associated therewith are facilitated.

20 Even more advantageous is the combination of sialic acid with established prebiotics such as galactooligosaccharides (GOS) and/or fructooligosaccharides (FOS) including inulin.

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

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

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

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

30 Furthermore, whereas some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to fall within the scope of the invention, and form different

embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor
5 with the necessary instructions for carrying out such a method or element of a method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an example of a means for carrying out the function performed by the element for the
10 purpose of carrying out the invention.

In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order to simplify the description
15 and facilitate understanding.

The invention will now be described by means of a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art without departing from the true spirit or technical teaching of the invention, the invention
20 being limited only by the terms of the appended claims.

Examples

Example 1: Metabolic engineering of an *E. coli* BL21(DE3) strain producing *N*-acetylneuraminic acid

Metabolic engineering was achieved by the mutagenesis and deletions of specific
25 endogenous genes and the genomic integration of heterologous genes. The genes *lacZ* and *araA* were inactivated by mutagenesis using mismatch-oligonucleotides as described by Ellis *et al.*, (Proc. Natl. Acad. Sci. USA 98: 6742-6746 (2001)).

Genomic deletions were generated according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). To prevent the degradation of *N*-acetylglucosamine the following genes were deleted from the genome of *E. coli* strain BL21 (DE3): *N*-acetylglucosamine specific PTS enzyme II (5 *nagE*), *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*), and glucosamine-6-phosphate deaminase (*nagB*). The whole *N*-acetylneuraminic acid catabolic gene cluster encoding *N*-acetylmannosamine kinase (*nanK*), *N*-acetylmannosamine-6-phosphate epimerase (*nanE*), *N*-acetylneuraminic acid aldolase (*nanA*) and the sialic acid permease (*nanT*) was also deleted. The genes *manX*, *manY* and *manZ*, 10 encoding a phosphoenolpyruvate-dependent phosphotransferase system facilitating the import of glucosamine, were also deleted. The *wzxC-wcaJ* genes were also deleted. The *wcaJ* gene encodes an UDP-glucose:undecaprenyl phosphate glucose-1-phosphate transferase catalyzing the first step in colanic acid synthesis (Stevenson *et al.*, J. Bacteriol. 1996, 178:4885-4893). In addition, the genes *fucI* and 15 *fucK* and *agaA* were deleted, encoding L-fucose isomerase, L-fucose kinase, and *N*-acetylgalactosamine-6-phosphate deacetylase, respectively.

The genomic integration of heterologous genes was achieved by transposition, using either the EZ-Tn5TM transposase (Epicentre, USA) or the hyperactive C9-mutant of the mariner transposase *Himar1* (Proc. Natl. Acad. Sci. 1999, USA 20 96:11428-11433). To produce EZ-Tn5 transposomes the gene of interest together with a FRT-site flanked antibiotic resistance marker (alternatively the resistance marker gene was flanked by *lox66-lox71* sites) was amplified. The resulting PCR-product carried at both termini the 19-bp Mosaic End recognition sites for the EZ-Tn5 transposase. For integration using *Himar1* transposase expression constructs 25 (operons) of interest were similarly cloned together with a FRT-site/*lox66-lox71*-site flanked by antibiotic resistance markers and transferred into the pEcomar vector, which encodes the hyperactive C9-mutant of the mariner transposase *Himar1* under the control of the arabinose-inducible promoter P_{araB}. All genes were codon-optimized for expression in *E. coli* and prepared synthetically by GenScript Corp.

30 The expression fragment <P_{tet}-*lacY*-FRT-*aadA*-FRT> (SEQ ID NO: 1) was integrated by using the EZ-Tn5 transposase. After successful integration of the gene for the lactose importer LacY from *E. coli* K12 TG1 (GenBank: ABN72583) the

resistance gene was eliminated from streptomycin resistant clones by the FLP recombinase encoded on plasmid pCP20 (Proc. Natl. Acad. Sci. 2000, USA 97:6640-6645). The *csc*-gene cluster (SEQ ID NO: 2) from *E. coli* W (GenBank: CP002185.1), comprising the genes for sucrose permease, fructokinase, sucrose
 5 hydrolase, and a transcriptional repressor (genes *cscB*, *cscK*, *cscA*, and *cscR*, respectively), enabling the strain to grow on sucrose as a sole carbon source, was also inserted in the genome. This cluster was integrated into the genome of the *E. coli* BL21(DE3) strain by transposition using plasmid pEcomar-*cscABKR*.

The resulting strain was further modified for the production of Neu5Ac by the
 10 genomic integration of the following expression cassettes: <P_{tet}-*slr1975-gna1-lox66-aacC1-lox71*> (SEQ ID NO: 3), <P_{tet}-*neuB-lox66-kanR-lox71*> (SEQ ID NO: 4) <P_{tet}-*slr1975-P_{t5}-neuB-FRT-dhfr-FRT*> (SEQ ID NO: 5), <P_{tet}-*glmS*-gna1-lox66-aacC1-lox71*> (SEQ ID NO: 6) and <P_{tet}-*ppsA-lox66-aacC1-lox71*> (SEQ ID NO: 7). Except
 15 removed in a stepwise manner from the genome (before the next round of gene integration) by introducing plasmid pKD-Cre (SEQ ID NO: 8) followed by selection on 2YT agar plates containing 100 µg·mL⁻¹ ampicillin and 100 mM L-arabinose at 30 °C. Resistant clones were subsequently transferred to 2YT agar plates lacking ampicillin as well as the selective antibiotic used for genomic integration. The eplates
 20 were incubated at 42 °C to cure the cells of the plasmid. Clones that were sensitive to ampicillin and the selective antibiotic were used for further experiments and modifications.

The gene *slr1975* (GenBank: BAL35720) encodes *Synechocystis* sp. PCC6803 *N*-acetylglucosamine 2-epimerase. The gene *gna1* (GenBank: NP_116637) encodes a
 25 glucosamine-6-phosphate acetyltransferase from *Saccharomyces cerevisiae*. The gene *neuB* (GenBank: AF305571) encodes a sialic acid synthase from *Campylobacter jejuni*. The gene *glmS** is a mutated version of the *E. coli* L-glutamine:D-fructose-6-phosphate aminotransferase gene (Metab Eng. 2005 May;7(3):201-14). The gene *ppsA* (GenBank: ACT43527) encodes the
 30 phosphoenolpyruvate synthase of *E. coli* BL21(DE3).

For the generation of <P_{tet}-*slr1975-gna1-lox66-aacC1-lox71*>, the genes *slr1975* and *gna1* were subcloned as an operon behind the constitutive promotor P_{tet} and

fused to the gentamycin resistance gene (flanked by lox66/lox71 sites) and inserted into the pEcomar vector by blunt-end ligation. The resulting expression cassette was integrated into the genome using vector pEcomar-*slr195-gna1-aacC1* and the hyperactive C9-mutant of the mariner transposase *Himar1* under the control of the

5 arabinose-inducible promoter P_{araB}.

For the generation of <P_{tet}-*neuB*-lox66-*kanR*-lox71>, *neuB* was cloned behind the constitutive promotor P_{tet} and fused to the kanamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome using the EZ-Tn5 transposase. For the generation of <P_{tet}-*slr1975*-P_{t5}-

10 *neuB*-FRT-*dhfr*-FRT>, the genes *slr1975* and *neuB* were separately subcloned behind the constitutive promoters P_{tet} and P_{t5}, respectively, and fused to the trimethoprim resistance gene (flanked by FRT sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

Expression cassette <P_{tet}-*glmS**-*gna1*-lox66-*aacC1*-lox71> was generated by

15 cloning *glmS** and *gna1* as an operon behind the constitutive promotor P_{tet}. This construct was further fused to the gentamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

For the generation of <P_{tet}-*ppsA*-lox66-*aacC1*-lox71>, the *ppsA* gene was cloned

20 behind the constitutive promoter P_{tet} and fused to the gentamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

Altogether, the cumulative genome modifications gave rise to the Neu5Ac-producing strain *E. coli* #NANA1.

25 **Example 2: Production of *N*-acetylneuraminic acid during a fed-batch fermentation**

The *E. coli* BL21 (DE3) strain #NANA1 was cultivated at 30°C in 3 L fermenters (New Brunswick, Edison, USA) starting with 1000 mL mineral salts medium containing 7 g·L⁻¹ NH₄H₂PO₄, 7 g·L⁻¹ K₂HPO₄, 2 g·L⁻¹ KOH, 0.3 g·L⁻¹ citric acid, 2

- g·L⁻¹ MgSO₄ × 7·H₂O, 5 g·L⁻¹ NH₄Cl₂ and 0.015 g·L⁻¹ CaCl₂ × 6·H₂O, supplemented with 1 mL·L⁻¹ trace element solution (54.4 g·L⁻¹ ammonium ferric citrate, 9.8 g·L⁻¹ MnCl₂ × 4·H₂O, 1.6 g·L⁻¹ CoCl₂ × 6·H₂O, 1 g·L⁻¹ CuCl₂ × 2·H₂O, 1.9 g·L⁻¹ H₃BO₃, 9 g·L⁻¹ ZnSO₄ × 7·H₂O, 1.1 g·L⁻¹ Na₂MoO₄ × 2·H₂O, 1.5 g·L⁻¹ Na₂SeO₃, 1.5 g·L⁻¹ NiSO₄ × 6·H₂O) and containing 2 % (m/v) sucrose as carbon source as well as the antibiotic zeocin (10 µg·mL⁻¹). Cultivation was started with a 2.5 % (v/v) inoculum from a pre-culture grown in the same sucrose-containing medium. The end of the batch phase was characterized by a rise in the dissolved oxygen level. A sucrose feed was applied immediately after leaving the batch phase. The 50 % (m/v) sucrose feed was supplemented with 2 g·L⁻¹ MgSO₄ × 7·H₂O, 0.015 g·L⁻¹ CaCl₂ × 6·H₂O and 1 mL·L⁻¹ trace element solution. A feeding rate of 9.0 to 11.0 mL·L⁻¹ was applied, referring to the starting volume. Aeration was maintained at 3 L·min⁻¹. Dissolved oxygen was maintained at 20–30 % saturation by controlling the rate of agitation. The pH was maintained at 7.0 by adding 25 % ammonia solution.
- High performance liquid chromatography (HPLC, Shimadzu) was used to detect Neu5Ac in the culture supernatant. The equipment comprised a UV-VIS detector at λ = 210 nm (SPD-10AVP, Shimadzu) and a Rezex ROA-organic acid H+ analytical column (300 x 7.8 mm) with an appropriate guard cartridge. Isocratic elution was performed in 5 mM H₂SO₄ at 50 °C was carried out at a flow rate of 0.5 mL·min⁻¹. The culture supernatant was centrifuged, filter-sterilized and heated at 95 °C for 5 min. After a final centrifugation, 5 µL of the sample was applied to the column. The concentration of *N*-acetylneuraminic acid was calculated from a standard curve using a commercially available standard (Carbosynth, Compton, UK). After incubation for 88 h, the final Neu5Ac titer in the culture supernatant was of 68.6 g·L⁻¹.
- Example 3: Generation and cultivation of single knock-out mutants in *E. coli* strain #NANA1 revealing improved Neu5Ac production capability**
- The *E. coli* BL21 (DE3) strain #NANA1 was further modified creating deletion mutants that removed or disrupted the genes *gltB*, *yjhC* and *ppc*. Strains #NANA1, #NANA1Δ*gltB*, #NANA1Δ*yjhC* and #NANA1Δ*ppc* were cultivated in 96-well plates. Therefore, single colonies of the strains were transferred from agar plates into microtiter plates containing 200 µL of the minimal medium described in Example 2

and were incubated for ~20 h at 30°C with vigorous shaking. Subsequently, 50 µL of the culture broth was transferred to deepwell 96-well plates (2.0 mL) containing 400 µL of minimal medium per well.

After an incubation for another 48 hours, cultivation was stopped and the quantity of
5 *N*-acetylneuraminic acid in the supernatant was determined by mass spectrometry
in multiple reaction monitoring (MRM) mode using an LC Triple-Quadrupole MS
detection system. Precursor ions are selected and analyzed in quadrupole 1,
fragmentation takes place in the collision cell using argon as the collision gas, and
fragment ions are selected in quadrupole 3. A 1-µL sample of *N*-acetylneuraminic
10 acid was injected into the HPLC instrument after diluting the culture supernatant
1:100 with LC/MS-grade water. The sample was separated on a XBridge Amide
HPLC column (3.5 µm, 2.1 × 50 mm (Waters, USA) with a XBridge Amide guard
cartridge (3.5 µm, 2.1 × 10 mm) (Waters, USA) at 50°C in acetonitrile:H₂O with 10
mM ammonium acetate at a flow rate of 400 µL·min⁻¹. Each separation lasted 240 s.
15 *N*-acetylneuraminic acid was analyzed by MRM in electrospray ionization (ESI)
positive-ionization mode. The mass spectrometer was operated at unit resolution. *N*-
acetylneuraminic acid forms an ion of m/z 309.2 [M+H]. The precursor ion of *N*-
acetylneuraminic acid was fragmented further in the collision cell into the fragment
ions m/z 292.20, m/z 274.15 and m/z 121.15. Collision energy, Q1 and Q3 Pre Bias
20 were optimized for each analyte individually. The quantification method was
established using a commercially available standard (Carbosynth, Compton, UK).

FIG. 3 shows the relative Neu5Ac yields of the cultivated strains. In comparison to
the parental strain, the value for strain #NANA1 was set to 100 %, and the single
knock-out mutants produced 20 % to 25 % more *N*-acetylneuraminic acid.

25 **Example 4: Purification of Neu5Ac from fermentation broth**

After termination of the fermentation, the biomass was separated from the
fermentation medium by ultrafiltration followed by using sequentially a winding
module filter (0.05 µm cut-off) (CUT membrane technology, Erkrath, Germany), and
a cross-flow filter (150 kDa cut-off) (Microdyn-Nadir, Wiesbaden, Germany). An

approximately 1 m³ cell-free fermentation medium was obtained containing more than 19 g·L⁻¹ sialic acid.

The cell-free liquid was then deionized by ion exchange chromatography. First, cationic contaminants were removed on a strong cationic exchanger in a volume of 200 L (Lewatit® S 2568 (Lanxess AG, Cologne, Germany) in H⁺ form. Using NaOH the obtained solution with a pH of about 1.5 was neutralized to 7.0. In a second step, anionic ions and undesired colorants were removed from the solution using the strong anionic exchanger Lewatit® S 6368 A (Lanxess AG, Cologne, Germany) in the chloride form. The ion exchanger had a bed volume of 200 L. Using a second filtration step on the cross-flow filter (150 kDa cut-off) (Microdyn-Nadir GmbH, Wiesbaden, Germany), precipitates originating from acidifying the solution were removed. For concentration of the sugar, the solution was nanofiltrated on a Dow Filmtec® NF270-4040 (INAQUA Vertriebsgesellschaft mbH, Mönchengladbach, Germany) to about ¼ of the Volume. The concentrated Neu5Ac solution was then further concentrated on a rotary evaporator to a concentration of about 400 g L⁻¹ or higher. Specific crystallization of the product was performed with a 10-fold excess of glacial acetic acid at 5 °C for 12-60 hours. The solid fraction was filtrated and washed with ethanol and dried at 40 °C. The dry crystallized product was further purified. Therefore, the dry product was solved in 2L H₂O per kg, and treated with activated charcoal (CAS-No: 7440-44-0, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The clarified solution, after separation from the charcoal, was concentrated by evaporation at 50 °C until it solidified. The solid material was mixed with 99% ethanol and incubated at 4 °C at least for 16 h. Afterwards the solid fraction was filtrated and dried at 40 °C. A crystalline, white product having a purity of more than 95% by means of area under curve of a chromatogram as determined by HPLC using a Rezex ROA-organic acid H⁺ column (Phenomenex, Aschaffenburg, Germany) was obtained.

Example 5: An alternative pathway for *N*-acetylneuraminic acid production

The *E. coli* BL21 (DE3) strain as described in example 1 ($\Delta lacZ$, $\Delta araA$, $\Delta nagABE$, $\Delta nanATEK$, $\Delta manXYZ$, $\Delta wcaJ$, $\Delta fucIK$, $\Delta agaA$, $lacY^+$, $cscABKR^+$) was further modified by integration of the expression cassette $\langle P_{tet}-glmS^*-gna1-lox66-aacC1-$

- lox71>, giving rise to a strain being able to synthesize *N*-acetylglucosamine (strain A). The strain A was modified to generate a strain for the production of *N*-acetylneuraminic acid. To this end, the expression constructs <P_{tet}-*slr1975*-P_{t5}-*neuB*-FRT-*dhfr*-FRT> (SEQ ID NO: 5) or <P_{tet}-*EcnanE*-P_{t5}-*neuB*-FRT-*dhfr*-FRT> (SEQ ID NO: 9) were individually integrated into the genome of strain A resulting in strains B and C, respectively. The *EcnanE* gene (GenBank: YP_003614592) encodes an *N*-acylglucosamine-6-phosphate 2-epimerase from *Enterobacter cloacae subsp. cloacae* ATCC 13047. All expression cassettes were integrated into the genome using the EZ-Tn5 transposase.
- Single colonies of these strains were transferred from agar plates into microtiter plates containing 200 µL of the minimal medium described in example 2 and were cultivated for ~20 h at 30 °C with vigorous shaking. Subsequently, 50 µL of the culture broth was transferred to deepwell 96-well plates (2.0 mL) containing 400 µL of minimal medium per well. After incubation of another 48 hours, cultivation was stopped and the *N*-acetylneuraminic acid level in the supernatants were determined by mass spectrometry.

- Neu5Ac production was only detectable in culture supernatants of strains B and C. In FIG. 4, the relative Neu5Ac production of the cultivated strains is shown. In comparison, the Neu5Ac production value of strain B was set to 100 %. Strain C produced about 7.5 % of Neu5Ac as compared to strain B.

Example 6: Composition of an infant formula containing Neu5Ac

Infant formula:

- Skimmed milk
- Vegetable oils (palm oil, rapeseed oil, sunflower oil)
- Human milk oligosaccharides
- L-Fucose
- N*-acetylneuraminic acid
- Skimmed milk powder
- Oil of *Mortierella alpine*
- Fish oil
- Calcium carbonate
- Potassium chloride
- Vitamin C
- Sodium chloride
- Vitamin E
- Iron acetate

Zinc sulphate
Niacin
Calcium-D-panthothenate
Copper sulphate
Vitamin A
Vitamin B1
Vitamin B6
Magnesium sulphate
Potassium iodate
Folic acid
Vitamin K
Sodium selenite
Vitamin D

CLAIMS

1. A non-naturally-occurring microorganism which is capable of producing Neu5Ac, wherein said non-naturally-occurring microorganism possesses a sialic acid biosynthesis pathway comprising at least one heterologous
5 enzyme, wherein the naturally occurring sialic acid catabolic pathway of the microorganism has been disabled, wherein at least one phosphoenolpyruvate:sugar phosphotransferase system for the import of a saccharide that is not used as a carbon source during fermentative production of Neu5Ac has been disabled, and wherein the microorganism can utilize an
10 exogenous carbon source present in the fermentation broth as a sole carbon source without using a phosphoenolpyruvate:sugar phosphotransferase system for the acquisition of said exogenous carbon source.
2. The non-naturally-occurring microorganism according to claim 1, wherein the sialic acid biosynthesis pathway comprises the enzymes glutamine--fructose-
15 6-phosphate aminotransferase, glucosamine-6-phosphate N-acetyltransferase, *N*-acetylglucosamine 2-epimerase, *N*-acetylneuraminic acid synthase, and a sugar phosphatase of the HAD-like superfamily.
3. The non-naturally-occurring microorganism according to claim 1 or 2, wherein
20 at least one of the enzymes of the sialic acid biosynthesis pathway is a heterologous enzyme.
4. The non-naturally-occurring microorganism according to any one of claims 1 to 3, wherein the naturally occurring sialic acid catabolic pathway has been disabled.
5. The non-naturally-occurring microorganism according to any one of claims 1
25 to 4, wherein one or more of the genes encoding an enzyme which is involved in the sialic acid catabolic pathway is deleted from the genome of the non-naturally-occurring microorganism, in that the expression of one or more of the genes encoding an enzyme which is involved in the sialic acid catabolic

pathway is impaired, or in that the nucleotide sequence of the protein coding region of at least one gene encoding an enzyme which is involved in the sialic acid catabolic pathway is amended such that the polypeptide being encoded by said altered protein-coding nucleotide sequence does not possess the enzymatic activity of the enzyme being encoded by the non-amended nucleotide sequence.

6. The non-naturally-occurring microorganism according to any one of claims 1 to 5, wherein the non-naturally-occurring microorganism has been genetically engineered to delete one or more of the genes selected from the group of genes encoding *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase, *N*-acetylneuraminic acid aldolase and sialic acid permease, in that the expression of one or more of these genes is impaired, or in that the nucleotide sequence of the protein coding region of at least one of these genes is amended such that the polypeptide encoded by said altered nucleotide sequence does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.
7. The non-naturally-occurring microorganism according to any one of claims 1 to 6, wherein the activities of one or more of the enzymes selected from the group consisting of *N*-acetylglucosamine-6-phosphate deacetylase and *N*-acetylglucosamine-6-phosphate deaminase has been abolished.
8. The non-naturally-occurring microorganism according to any one of claims 1 to 7, wherein the non-naturally-occurring microorganism has been genetically engineered to abolish *N*-acetylglucosamine-6-phosphate deacetylase and/or *N*-acetylglucosamine-6-phosphate deaminase activity by deleting one or both genes encoding these enzymes, by impairing expression of one or both of these genes, or by mutating the protein-coding region of one or both genes such that the polypeptide being encoded by each altered nucleotide sequence does not possess the enzymatic activity of the enzyme encoded by the non-amended nucleotide sequence.

9. The non-naturally-occurring microorganism according to any one of claims 1 to 8, wherein at least one phosphoenolpyruvate (PEP)-dependent, sugar transporting phosphotransferase system for the import of a carbohydrate has been disabled.
- 5 10. The non-naturally-occurring microorganism according to any one of claims 1 to 9, wherein the non-naturally-occurring microorganism comprises a saccharide/H⁺ symporter, preferably a saccharide/H⁺-symporter selected from the group consisting of a sucrose proton symporter, a lactose proton symporter and a glucose proton symporter.
- 10 11. The non-naturally-occurring microorganism according to any one of claims 1 to 10, wherein the non-naturally-occurring microorganism possesses an enhanced PEP biosynthesis as compared to the wild-type microorganism, preferably due to the overexpression of PEP synthase.
- 15 12. The non-naturally-occurring microorganism according to any one of claims 1 to 11, wherein the non-naturally-occurring microorganism does lacks one or more selected from the group consisting of a functional PEP carboxylase, a functional glutamate synthase, a functional Wzx protein, a functional UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase, a functional β -galactoside permease, a functional β -galactosidase, a functional YjhC protein, a functional fucose isomerase, a functional fuculokinase, and a functional *N*-acetylglutamine aminoacylase.
- 20 13. The non-naturally-occurring microorganism according to any one of claims 1 to 12, wherein the non-naturally-occurring microorganism possesses an enhanced glutamine synthesis as compared to the wild-type microorganism, preferably due to the overexpression of glutamine synthase.
- 25 14. Use of a non-naturally-occurring microorganism according to any one of claims 1 to 13 for the production of Neu5Ac.

15. A method for producing Neu5Ac by fermentation using a non-naturally-occurring microorganism able to produce Neu5Ac, the method comprising the following steps:
- 5 a) providing a non-naturally-occurring microorganism of any one of claims 1 to 13;
- b) cultivating the non-naturally-occurring microorganism in a fermentation broth and under conditions permissive for the non-naturally-occurring microorganism to produce Neu5Ac; and
- c) optionally, recovering the Neu5Ac from the fermentation broth.
- 10 16. The method according to claim 15, wherein the fermentation broth contains a carbon source for growing the non-naturally-occurring microorganism, the carbon source preferably being selected from the group consisting of glucose, xylose, sucrose, fructose, lactose, glycerol, syngas and combinations thereof.
- 15 17. Use of Neu5Ac as produced by a method according to claim 15 or 16 for the manufacture of a nutritional composition.
18. A nutritional composition containing Neu5Ac which is produced by a method according to claim 15 or 16.
19. The nutritional composition according to claim 18, wherein said composition
20 also contains at least one HMO, preferably at least one neutral HMO and/or at least one sialylated HMO.
20. The nutritional composition according to claim 18 or 19, wherein the nutritional composition also contains a probiotic microorganism.
21. The nutritional composition according to any one of claims 18 to 20, wherein
25 the nutritional composition is one selected from the group consisting of medicinal formulations, infant formula and dietary supplements.

22. The nutritional composition according to any one of claims 18 to 21, wherein the nutritional composition is present in liquid form, preferably in form of a concentrate or ready to drink, or in solid form, preferably in form of a powder, granules, flakes or pellets.
- 5 23. An infant formula containing Neu5Ac which is produced by a method according to any one of claims 1 to 13.