Advanced microbiome therapeutics engineered to produce serotonin in vivo

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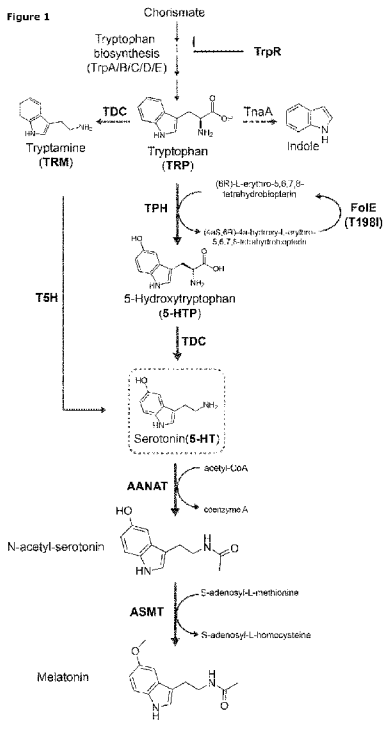
(54) Title: ADVANCED MICROBIOME THERAPEUTICS ENGINEERED TO PRODUCE SEROTONIN IN VIVO



(57) Abstract: The invention provides a composition for use as a medicament, comprising cells of a recombinant microorganism capable of producing increased amounts of one or more of 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT) and tryptamine (TRM) as compared to the non-recombinant microorganism from which it was derived. The composition finds use in preventing and/or treating TRM-; 5-HTP-, or 5-HT-related disorders of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI) and metabolism in a mammal, and may be orally administered to a mammal in need thereof. Additionally, a composition comprising cells of a recombinant microorganism capable of producing melatonin is provided for use as a medicament, such as for treatment of depression, dementia, cancer and sleep disorder.

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Title: Advanced microbiome therapeutics engineered to produce serotonin in vivo

FIELD OF THE INVENTION

The invention provides a composition for use as a medicament, comprising cells of a recombinant microorganism capable of producing increased amounts of one or more of 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT) and tryptamine (TRM) as compared to the non-recombinant microorganism from which it was derived. The composition finds use in preventing and/or treating TRM-; 5-HTP-, or 5-HT-related disorders of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI) and metabolism in a mammal, and may be orally administered to a mammal in need thereof. Additionally, a composition comprising cells of a recombinant microorganism capable of producing melatonin is provided for use as a medicament.

BACKGROUND OF THE INVENTION

The biogenic monoamine serotonin (5-hydroxytryptamine, 5-HT) is crucial for neurotransmission and many other functions throughout the body. Up to 95% of the serotonin in the body is produced in the gastrointestinal tract. Serotonin is derived from tryptophan (TRP) in a two-step reaction, first by a tryptophan hydroxylase (TPH) to form the 5-hydroxytryptophan (5-HTP) intermediate and then by tryptophan decarboxylase (TDC), as illustrated in figure 1. In humans, tryptophan hydroxylase is the rate-limiting step of 5-HT biosynthesis and is made in two isoforms, TPH1 and TPH2. TPH2 is produced in neurons across the body, while TPH1 is predominately expressed in neuroendocrine cells in the gut.

In the GI tract, serotonin regulates intestinal motility, cell turnover, and homeostasis. In the brain, an imbalance in the serotonin level is linked to anxiety and depression. Treatment includes strategies for elevating serotonin in a patient suffering from any of the wide range of anxiety- and depression-related diseases.

Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed for the treatment of depression. They are believed to increase the extracellular level of the neurotransmitter serotonin by limiting its reabsorption (reuptake) into the presynaptic cell, increasing the level of serotonin in the synaptic cleft available to bind to the postsynaptic receptor. A sub-group of patients present symptoms that are SSRI-treatment resistant (TRD). A treatment of TRD patients, currently under clinical trial in mammalian models, is based on a combination therapy of an SSRI (serotonin re-uptake inhibitor) and the serotonin precursor, 5-hydroxytryptophan (5-HTP). This has undesirable consequences, due to the rapid uptake of administered 5-HTP, leading to

sudden spikes in 5-HT levels. Efforts to address this problem currently focus on combination therapies employing slow-release forms of 5-HTP.

Intestinal bacteria play a crucial role in gastrointestinal (GI) health and homeostasis. Beyond modulating gut homeostasis, intestinal microbes are thought to participate in a bidirectional signaling pathway with the enteric nervous system (ENS) and the central nervous system (CNS) along the gut-brain axis. Resident microbiota in the gut are known to send signals to host mucosal endocrine cells to maintain gut 5-HT content and 5-HT plasma levels, rather than themselves providing a significant supply of 5-HT. More recently, it has been shown that oral ingestion of certain bacteria in animal models leads to changes in host physiology and behavior, implicating a role of these microbes in gut-brain communication.

In the Hippocampus, striatum, cortex, and dentate gyrus of the brain, serotonin-based biosynthesis pathways, serotonin-based receptors, and serotonin-based signaling pathways are implicated in memory, cognitive/age-related spatial learning, and memory formation. As serotonergic afferent neurons from the gastrointestinal tract alters dorsal motor nucleus of the vagus and nucleus of the *tractus solitarius* through the vagus nerve, intestinal 5-HT is implicated in neuronal memory, cognition, and learning.

In view of the key role of TRM and 5-HT in mammalian homeostasis and medical conditions arising from insufficient 5-HT levels, there exists a need for new treatments for elevating the level of 5-HT in patients, in particular in those suffering from anxiety-related diseases, as well as TRD.

The signaling molecule melatonin is a highly pleiotropic molecule that is released as a hormone of the pineal gland predominantly during night. Melatonin secretion decreases during aging, but reduced melatonin levels are also observed in various diseases, such as types of dementia, some mood disorders, severe pain, cancer, and diabetes type 2. Melatonin dysfunction is frequently related to deviations in amplitudes, phasing, and coupling of circadian rhythms. Accordingly there exists a need for treatments that will elevate the level of melatonin in patients in need thereof.

SUMMARY OF THE INVENTION

In a first embodiment, the invention provides a composition for use as a medicament, wherein said composition comprises cells of a recombinant microorganism, and wherein said microorganism comprises one or more recombinant nucleic acid molecules encoding one of more proteins selected from:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),

(b) tryptophan decarboxylase (EC 4.1.1.28), and
(c) tryptamine 5-hydroxylase (EC:1.14.-.-)

The cells are therefore capable of producing increased amounts of one or more of 5-hydroxytryptophan, 5-hydroxytryptamine and tryptamine as compared to cells of the non-recombinant microorganism from which it was derived.

In a second embodiment, the invention provides a recombinant bacterial cell comprising one or more recombinant nucleic acid molecules or transgenes encoding one of more proteins selected from:
(a) tryptophan 5-hydroxylase (EC 1.14.16.4),
(b) tryptophan decarboxylase (EC 4.1.1.28), and
(c) tryptamine 5-hydroxylase (EC:1.14.-.-)
wherein said microorganism is devoid of genes capable of expressing *trp* operon repressor protein and tryptophanase (EC:4.1.99.1). The cell is therefore capable of producing increased amounts of one or more of 5-hydroxytryptophan, 5-hydroxytryptamine and tryptamine as compared to a cell of the non-recombinant microorganism from which it was derived.

Preferably, the recombinant bacterial cell is selected from among *Escherichia, Bacteroides, Clostridium, Feacalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Staphylococcus* and *Bacillus.*

In a third embodiment the invention provides a method for preventing and/or treating a TRM-; 5-HTP-, 5-HT-; or melatonin-related disorder in a subject, the method comprising administering to the subject diagnosed with a TRM-; 5-HTP-; 5-HT-; or melatonin-related disorder recombinant bacteria engineered to express one or more:
(a) tryptophan 5-hydroxylase (EC 1.14.16.4),
(b) tryptophan decarboxylase (EC 4.1.1.28), or
(c) tryptamine 5-hydroxylase (EC:1.14.-.-)
The cells are therefore capable of producing increased amounts of one or more of 5-hydroxytryptophan, 5-hydroxytryptamine and tryptamine as compared to cells of the non-recombinant microorganism from which it was derived.

The TRM-; 5-HTP-, 5-HT-; or melatonin-related disorder may be a disorder of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI) or metabolism in a mammal.

In a fourth embodiment the invention provides a recombinant microorganism comprising one or more recombinant nucleic acid molecules or transgenes, wherein said microorganism comprises recombinant nucleic acid molecules encoding:
(a) tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or
(b) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-), and
recombinant nucleic acid molecules encoding:

(c) serotonin acetyltransferase (AANAT) EC 2.3.1.87 and
(d) acetylserotonin O-methyltransferase (ASMT) (EC 2.1.1.4),
wherein the cells are capable of producing melatonin. The cells are therefore capable of producing melatonin in amounts that are increased as compared to cells of the non-recombinant microorganism from which it was derived.

In a fifth embodiment, the invention provides a composition for use as a medicament, wherein said composition comprises cells of a recombinant microorganism capable of producing melatonin according to the fourth embodiment; suitable for oral delivery in a patient suffering from a melatonin-deficient or melatonin-related disorders, such as a the following melatonin-related disorders: circadian rhythm disturbance, insomnia, jet lag, autism; dementia, mood disorders, severe pain, cancer, and diabetes type 2.

DESCRIPTION OF THE INVENTION

Brief description of the figures:

Figure 1 Cartoon showing the catalytic steps of the 5-HT and melatonin biosynthetic pathway. TrpR (tryptophan operon repressor protein); TnaA (tryptophanase); FolE (encodes mutant GTP cyclohydrolase 1 (T198I)) TRP (tryptophan); TRM (tryptamine); 5-HTP (5-Hydroxytryptophan); 5-HT (serotonin); TPH (tryptophan hydroxylase); TDC (tryptophan decarboxylase); T5H (tryptamine 5-hydroxylase); serotonin acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT).

Figure 2 [**A**]: Histogram showing titers of TRM (tryptamine); 5-HTP (5-Hydroxytryptophan); 5-HT (serotonin) measured in culture media derived from cultures of cells of six *E. coli* Nissle strains engineered to co-express respectively: TPH and TDC genes encoding Human TPH1 and rice TDC (H1R); human THP2 (H2) and rice TDC (H2R); human THP2 (H2) and *Cataranthus roseus* TDC (H2C); human THP2 (H2) and Guinea pig TDC (H2G); mouse TPH1 (M1) and rice TDC (M1R); and mouse THP2 (M2) and rice TDC (M2R). [**B**]: Histogram showing titers of TRM (tryptamine); 5-HTP (5-

Hydroxytryptophan); 5-HT (serotonin) measured in culture media derived from 5-HTP-fed cultures of cells of three *E. coli* Nissle strains engineered to co-express respectively: TPH and TDC genes encoding human THP2 (H2) and rice TDC (H2R); human THP2 (H2) and *Cataranthus roseus* TDC (H2C); human THP2 (H2) and Guinea pig TDC (H2G).

Figure 3 Histogram showing titers of TRM (tryptamine); 5-HTP (5-Hydroxytryptophan); and 5-HT (serotonin) measured in culture media following cultivation of an *E. coli* Nissle strain (EcNΔ2 + pUC-H2R) co-expressing TPH and TDC in growth media supplemented with the substrate, in the range of 10 – 100 mg tryptophan/L.

Figure 4 [A] Graph showing cell density of cultures of four *E. coli* Nissle strains, measured as units of OD_{600nm}. The strains are EcNΔ2 + pUC-H2R; EcNΔ3 + pUC-H2R; EcNΔ2 + pMUT-H2R, each co-expressing human TPH2 (H2) and rice TDC (R); and EcN + pMUT empty plasmid. EcNΔ2 has deletions: ΔtrpR, ΔtnaA; EcNΔ3 has deletions ΔtrpR, ΔtnaA, ΔinfA. [**B**] Graph showing cell density of cultures of nine *E. coli* Nissle strains co-expressing human TPH2 (H2) or mouse TPH1 (M1) with rice TDC (R); and control strains comprising empty plasmids, measured as units of OD_{600nm}. [**C**] Graph showing per cent kanamycin resistant colonies detected in cultures of *E. coli* Nissle strains comprising pUC-based or pMUT-based serotonin production plasmids measured over a period of 100 generations of growth without antibiotic selection. At each time point, cells were plated on LB Agar plates +/- kanamycin, and ratios of colonies were calculated (n=6). [**D**] Histogram showing titers of 5-HT (serotonin) measured in culture media following cultivation of *E. coli* Nissle strains co-expressing human TPH2 (H2) and rice TDC (R) cloned in pUC (pUC-H2R) or pMUT (pMUT-H2R) plasmids for 0-100 cell generations.

Figure 5 [A] Histogram showing titers of TRM (tryptamine) and 5-HT (serotonin) measured in culture media following cultivation of *E. coli* Nissle strain N; NΔ2 and "oN" co-expressing human TPH2 and rice TDC (H2R), or mouse TPH1 and rice TDC (M1R) operons in pUC or pMUC plasmids, or carrying an empty pMUT plasmid. [**B**] Histogram showing titers of TRM (tryptamine), 5-HTP (5-hydroxytryptophan) and 5-HT (serotonin) measured in culture media following cultivation of *E. coli* Nissle "oN" strains expressing mouse TPH1 (oN10 = oN + pMUTM1); rice TDC (oN11 = oN + pMUTR); mouse TPH1 and rice TDC (oN14 = oN + pMUTM1R) or carrying an empty plasmid (oN9 = pMUT). All data shown are mean values with error bars of standard deviation from n=3 biological replicates [**C**] and [**D**] left panels: Histogram showing titers of TRP (tryptophan); TRM (tryptamine); 5-HTP (5-Hydroxytryptophan); and 5-HT (serotonin) measured in culture media following cultivation of an *E. coli* Nissle strain comprising plasmids pMUT-H2R and pMUT-M1R functionally linked to RBS sequences having a range of translation strengths. Right panels: 2-Dimensional diagram showing yields of 5-HT; 5-HTP and tryptamine yields in cultivation medium measured for *E. coli* Nissle strain comprising a plasmid

pMUT-H2R functionally linked to an RBS sequence having a range of translation strengths, x-axis corresponds to RBS strength for TPH gene; the y-axis corresponds to RBS strength for TDC; crossed out boxes indicate combinations not tested. [**E**] Cartoon showing genetic modifications to *E. coli* Nissle genome and genetic structure of the plasmids tested in [B]. [F] Histogram showing titers of 5-HTP measured in culture media following cultivation of *E. coli* Nissle strain NΔ2 (=folE WT) or oN (=folE(T1981I), each comprising the human TPH2 pMUT plasmid.

Figure 6 [**A**] Histogram showing titers of 5-HT (serotonin) and TRM (tryptamine) measured in culture media following cultivation of *E. coli* Nissle strain NΔ2 or strain oN each co-expressing human TPH2 and rice TDC (H2R) genes functionally linked to RBS sequences of different translation strengths cloned in a pUC plasmids. [**B**] Histogram showing titers of 5-HT (serotonin) and TRM (tryptamine) measured in culture media following cultivation of *E. coli* Nissle strain NΔ2 or strain oN each expressing a human TPH2 or mouse TPH1 gene in combination with a rice TDC (H2R) gene functionally linked to RBS sequences of different translation strengths cloned in pMUT plasmids. [**C**] Graph showing theoretical 5-HT concentration in the mouse gut relative to the abundance of 5-HT producing bacteria measured as a per cent of the total gut microbiome population. Theoretical physiological levels of 5-HT in the gut lie in the range of 5-30 mg/L as indicated. The relative abundance of 5-HT producing bacteria required to produce 5-HT within the therapeutically effective range is plotted for bacteria having an *in vivo* 5-HT production rate (P) of 0.1 to 10 mg/L*OD_{630}*h.

Figure 7 [**A**] Image of thin sections of mid-colon derived from mice 24 hours after last oral gavage with *E. coli* strains oN9, oN11, oN14 and control (PBS), where the sections were stained with DAPI and anti-GFP antibody. [**B**] Graph showing the kanamycin-resistant, GFP positive bacterial concentration in the duodenum, jejunal-ileal junction, cecum, proximal colon, distal colon, and fecal matter derived from mice 24 hours after last oral gavage with either *E. coli* strain oN9, oN11, or oN14, or control (PBS). Samples plated on LB Agar plates plus kanamycin and colonies counted measured as colony forming units (CFU) per gram of fecal matter (n=6). Data points shown at y=0 were below the level of detection of 10^3 CFU/gram.

Figure 8 Histogram showing [**A**] tryptophan and [**B**] 5-HT concentration measured as mg/L in the serum, mg/g biomass in cleaned homogenized colon tissue, mg/g biomass of fecal material (n=7-8) derived from mice 24 hours after last oral gavage with either *E. coli* strain oN9, oN11, or oN14, or control (PBS). All p-values shown are from one-way ANOVA with Tukey's *post hoc* correction for multiple comparison testing. All other comparisons not shown were not significant with $p>0.05$. Data are mean values with error bars as standard error of the mean. [**C**] Image of thin sections of mid-colon derived

from mice 24 hours after last oral gavage with *E. coli* strains oN9, oN11, oN14 and control (PBS), where the sections were stained with DAPI and anti-serotonin antibody, where the scale bar is 100 μm.

Figure 9 [**A**] Histogram showing relative expression of MUC2, TPH1, TPH2, and SERT gene expression levels, measured as relative mRNA by ΔΔCT (n=6), in colonic tissue derived from mice 24 hours after last oral gavage with either *E. coli* strain oN9, oN11, or oN14, or control (PBS). Data are mean values with error bars as standard error of the mean. [**B**] Image of thin sections of mid-colon derived from mice 24 hours after last oral gavage with *E. coli* strains oN9, oN11, oN14 and control (PBS), where the sections were stained with DAPI and anti-Muc-2 antibody, where the scale bar is 400 μm. The arrow indicates increased MUC2 signal in oN14.

Figure 10 Histograms [(**a**) – (**e**)] showing relative expression of 5-HT receptors HTR1B HTR1D, HTR3, HTR4, and HTR7 in the colon by RT-PCR (n=6), in colonic tissue derived from mice 24 hours after last oral gavage with either *E. coli* strain oN9, oN11, or oN14, or control (PBS). Histograms (**f**) showing total gastrointestinal transit time from mouth to anus using a 6% Carmine Red solution (Methods) (n=7-8) in correspondingly treated mice cohorts. All p-values shown are from one-way ANOVA with Tukey's post hoc correction for multiple comparison testing. All other comparisons not shown were not significant with $p>0.05$. Data are mean values with error bars as standard error of the mean.

Figure 11 Histograms showing (**a**) time spent immobile of mice during the last four minutes of a six-minute Forced Swim Test (FST) (n=9-10); (**b**) number of fecal pellets (# of fecal boli) produced during a 10-minute Open Field Test (OFT) (n=7-10); (**c**) average total distance traveled by mice during the 10-minute OFT (n=7-10); (**d**) time spent in both the inner 40 cm x 40 cm zone or outer 10 cm ring during OFT (n=7-10); and (**e**) representative tracking pattern of a single mouse from each mouse cohort after oral gavage with either *E. coli* strain oN9, oN11, or oN14, or control (PBS). All p values shown are from one-way ANOVA with Tukey's post hoc correction for multiple comparison testing. All other comparisons not shown were not significant with $p>0.05$. Data are mean values with error bars as standard error of the mean.

Figure 12 Histograms showing (**a**) number of fecal boli produced during a 10-minute Open Field Test (OFT) (n=7-10); (**b**) time spent by mice in the inner 40 cm x 40 cm zone during OFT (n=7-10); (**c**) average distance traveled by mice in the inner 40 cm x 40 cm zone during the 10-minute OFT (n=7-10); (**d**) number of entries by mice into the inner 40 cm x 40 cm zone during the 10-minute OFT (n=7-10); (**e**) average total distance traveled by mice during the 10-minute OFT (n=7-10); (**f**) time spent immobile of mice during the last four minutes of a six-minute Forced Swim Test (FST) (n=9-10);

as measured for each mouse cohort after 21 days administration of Gavage: PBS/IP: saline (negative control); Gavage: PBS/ IP: Fluoxetine (SSRI positive comparator), Gavage: *E. coli* strain oN14/ IP: saline (oN14 positive comparator); and Gavage: *E. coli* strain oN14/IP: Fluoxetine (positive combination comparator). All p values shown are from one-way ANOVA with Tukey's post hoc correction for multiple comparison testing. All other comparisons not shown were not significant with $p>0.05$. Data are mean values with error bars as standard error of the mean.

Figure 13 Histograms showing (**A**) 5-HT concentration and (**B**) tryptophan concentration in peripheral plasma samples, C) 5-HT concentration and D) 5-HTP concentration in urine samplesfrom mice after oral gavage with either *E. coli* strain oN10 or *E. coli* strain (EcN-control) lacking TPH expression; ($n=8$, $p<0.001$ by two-tailed t-test for plasma 5-HT).

Figure 14 Cartoon showing the structure of plasmids for expression of a serotonin pathway in *S. cerevisiae* (pSc-1 to pSc-3). RTDC=tryptophan decarboxylase gene (Rice); ck-TDC=tryptophan decarboxylase gene (*Candidatus Koribacter versatilis* Ellin345); TPH=tryptophan 5-hydroxylase 1 (mouse).

Figure 15 (**A**) Histogram showing titers of 5-HT (serotonin) measured in culture media following cultivation of *S. cerevisiae* with or without plasmids expressing mouse TPH1 and and TDC genes from either rice (Sc-1 and Sc-2) or from *Candidatus Koribacter versatilis* Ellin345 (Sc-3). (**B**) Histogram showing titers of 5-HT (serotonin) and TRM (tryptamine) measured in culture media following cultivation of *E. coli* Nissle strain oN co-expressing mouse TPH1 and TDC genes from either rice (oN14) or from *Candidatus Koribacter versatilis* Ellin345 (M1ck). (**C**) Graph showing cell density of cultures of four *E. coli* Nissle strains, measured as units of OD_{600nm}. oN control contains pMh-empty, oN14 contains pMUT14-5HT, M1R (trc) = mouse TPH1 and rice TDC expressed from *trc* promoter, M1ck (trc) = mouse TPH1 and ck-TDC expressed from *trc* promoter [SEQ ID No.: 112].

Figure 16 Cartoon showing the structure of a plasmid for expression of a serotonin pathway in *B. subtilis* (pBS-M1Rf). R-TDC=tryptophan decarboxylase gene (Rice); TPH=tryptophan 5-hydroxylase 1 gene (mouse), FolE=mutant GTP cyclohydrolase I gene (GCH1).

Abbreviations, terms and definitions:

By "non-recombinant microorganism from which it was derived" is meant a microorganism which does not comprise the recombinant nucleic acid molecules.

By "producing increased amounts" is meant compared to the non-recombinant organism without the recombinant nucleic acid molecules. For example, recombinant microbial cells expressing the recombinant enzymes of the claims are compared with microbial cells that do not comprise nucleic acid molecules for the enzymes in the claims. The cells with the recombinant nucleic acids encoding the enzymes will produce increased amounts of one or more of 5-hydroxytryptophan, 5-hydroxytryptamine and tryptamine as a result of expression of the encoded nucleic acids which express enzymes in the biosynthesis pathway according to Figure 1.

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity calculations are preferably automated using the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Multiple sequence alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

Devoid of a gene encoding a functional protein: a microorganism that is devoid of a gene capable of expressing a functional protein (e.g. tryptophan repressor or tryptophanase) is a microorganism that either lacks the respective gene, or the gene id

is modified (e.g inactivated) such that it is not able to express a functional protein. A range of genetic modifications are suitable for inactivating a gene, including the deletion of the gene (knockout) from the genome of a microbial cell; deletion of its cognate regulatory sequences (e.g. promoter); substitution of, or addition of, at least one nucleotide leading to a loss of expression of a functional polypeptide encoded by the gene. Where the encoded polypeptide is an enzyme, the genetic modification leads to a loss of detectable enzymatic activity of the respective polypeptide in the microbial cell.

Genome: is the genetic material present in a cell or organism; said genome comprising all of the information needed to build and maintain that cell or organism; and includes the genetic material in both chromosome(s) and plasmid(s) present within the cell or organism.

Mutant GTP cyclohydrolase I (EC 3.5.4.16): herein abbreviated as mutant GCH1, is to be understood as a mutant GCH1 that provides for an increased hydroxylation activity of a tryptophan 5-hydroxylase (EC 1.14.16.4) of at least 2-fold (for example: at least 3-fold, or 4-fold, or 5-fold, 6-fold or, 7-fold or, 8-fold or, 9-fold or, 10-fold), as compared to a non-mutant GCH1, as measured by 5-hydroxytryptophan yield. The non-mutant GCH1 can be the parent enzyme from which the mutant was derived, whereby mutation(s) found in the mutant confer the increased activity.

Native gene: endogenous gene in a microorganism cell genome, homologous to host microorganism.

RBS: **R**ibosomal **B**inding Site is a sequence of nucleotides upstream of the start codon of an mRNA transcript that is responsible for the recruitment of a ribosome during the initiation of protein translation.

Ribosome Binding Site (RBS) Calculator: provides a method for either predicting or controlling translation initiation rates (TIRs) in bacteria. When used to control translation of a given coding sequence, the RBS calculator generates synthetic DNA sequences that will result in a defined translation initiation rate (and therefore, protein expression strength). For any promoter sequence (controlling transcription strength), many different RBS strengths can be designed to regulate the amount of protein produced from this transcript (Salis at al., 2009).

RBS strength scale and units: The output values range from 1 to 1000000 on a linear scale with arbitrary units that were experimentally validated using fluorescent protein abundance as a measure of expression strength (Salis at al., 2009). Designed sequences of a particular strength are not unique, i.e. different nucleotide sequences can encode an RBS having the same RBS strength. Furthermore, RBS strength is always context-dependent, therefore a designed sequence of a defined strength is only valid for the coding sequence it was calculated for. The minimum nucleotide sequences defining RBS

strength are 35 base pairs upstream and 50 base pairs downstream of the start codon (ATG) of a coding sequence.

Transgene: a gene or genetic material that has been transferred naturally or by any of a number of genetic engineering techniques from one organism to another. The transgene that is transferred to the recipient can be from other individuals of the same species or even from unrelated species.

Detailed description of the invention:

I: In a first aspect, the invention provides a composition for use as a medicament, comprising cells of a recombinant microorganism, wherein said microorganism comprises one or more transgenes or recombinant nucleic acid molecules encoding one of more proteins selected from:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),
(b) tryptophan decarboxylase (EC 4.1.1.28), and
(c) tryptamine 5-hydroxylase (EC:1.14.-.-),

The cells of said microorganism are therefore capable of producing increased amounts of one or more of 5-hydroxytryptophan (5-HT), 5-hydroxytryptamine (5-HTP) and tryptamine (TRM) as compared to cells of the non-recombinant microorganism from which it was derived.

In a further aspect thereof, said one or more recombinant nucleic acid molecules encode proteins selected from the group:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),
(b) tryptophan decarboxylase (EC 4.1.1.28),
(c) tryptamine 5-hydroxylase (EC:1.14.-.-),
(d) tryptophan 5-hydroxylase (EC 1.14.16.4) and tryptophan decarboxylase (EC 4.1.1.28), and
(e) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-).

In a further aspect thereof, said composition is for use as a therapeutic in preventing and/or treating TRM-; 5-HTP-, or 5-HT-related disorders of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI); hormonal imbalance, metabolic disease, non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH), diabetes in a mammal.

More specifically, TRM-, 5-HTP or 5-HT homeostasis of the CNS treated by the composition of the invention include anxiety- and depression-related behavior, as well as memory-, cognition-, and psychiatric-disorders; in particular generalized anxiety

disorder, phobia disorder, social anxiety disorder, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, chronic stress disorder, separation and situational anxiety, age-related memory decline, and disorders in spatial memory formation, alertness, focus, learning and cognition, autism, migraine and immune-related disorders. TRM- or 5-HT related therapies of the GI that are treated by the composition of the invention include motility disorders, metabolic syndrome, obesity, weight-control, inflammation-associated sickness, inflammatory bowel disease, irritable bowel syndrome (IBS); celiac disease, diverticular disease, and colorectal cancer.

In another aspect the invention provides a composition for use as a medicament comprising a recombinant microorganism, wherein the microorganism comprises both one or more transgenes or recombinant nucleic acid molecules encoding:
(a) tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or
(b) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-),

and additionally transgenes or recombinant nucleic acid molecules encoding:

(c) serotonin acetyltransferase (AANAT) EC 2.3.1.87 and
(d) acetylserotonin O-methyltransferase (ASMT) (EC 2.1.1.4),
wherein the cells are capable of producing melatonin. The cells are therefore capable of producing melatonin in amounts that are increased as compared to cells of the non-recombinant microorganism from which it was derived.

In a further aspect thereof, the invention provides a composition for use as a medicament, wherein said composition comprises cells of a recombinant microorganism capable of producing melatonin; suitable for oral delivery in a patient suffering from a melatonin-deficient or melatonin-related disorders, such as the melatonin-related disorders: circadian rhythm disturbance, insomnia, jet lag, autism; dementia, mood disorders, severe pain, cancer, and diabetes type 2.

In a further aspect thereof, said composition is for oral administration to a mammal in need thereof.

In a further aspect thereof, the microorganism in said composition is a live facultative anaerobic gut bacterium or a yeast (Ianiro G et al., 2014), preferably a commensal or probiotic strain, characterized by the ability to survive and/colonize one or more regions of the mammalian gut. For example the bacterium is one selected from the genus *Escherichia, Bacteroides, Clostridium, Feacalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Lactococcus, Bifidobacterium,*

Enterococcus, Streptococcus, Pediococcus, Leuconostoc, *Staphylococcus* and *Bacillus.* Suitable species of bacterium include *Escherichia coli* (e.g. strain Nissle*), Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, Bifidobacterium longum* and *Bifidobacterium adolescentis.* Additionally, a suitable microorganism in said composition may be selected from microbial species identified by Li et al., 2014, and Zou et al., 2019, whose teaching is incorporated by reference.

In a further aspect thereof, the microorganism in said composition comprises a nucleic acid sequence encoding tryptophan 5-hydroxylase (TPH) (EC 1.14.16.4), which catalyzes the conversion of L-tryptophan + tetrahydrobiopterin + O(2) => 5-hydroxy-L-tryptophan + 4a-hydroxytetrahydrobiopterin by hydroxylating tryptophan at the C5 position (figure 1). The assay and quantification of 5-hydroxy-L-tryptophan yield are described in 1.1.2 and 1.1.3, respectively. Following oral administration to a mammal, the microorganism in said composition, is capable of expressing the TPH enzyme in the mammalian gut such as to produce and secrete 5-HTP (figure 2A). Both of the isoforms of tryptophan hydroxylase, TPH1 and TPH2, when expressed in a microorganism of the invention are suitable for producing 5-HTP; as well as 5-HT when co-expressed with a TDC having EC 4.1.1.28 (figure 2A). However, in a preferred embodiment the expressed TPH enzyme in the microorganism in the composition, for administration to the mammalian gastrointestinal tract, is TPH1 due to the low oxygen environment of the gut and TPH1's higher oxygen binding affinity, as compared to TPH2.

The microorganism in said composition may, either additionally or alternatively, comprise a nucleic acid molecule encoding tryptophan decarboxylase (TDC) (EC 4.1.1.28), which catalyzes the conversion of 5-hydroxy-L-tryptophan => 5-hydroxytryptamine + CO_2 (Figure 1). The assay and quantification of 5-hydroxytryptamine yield is described in 1.1.2 and 1.1.3. A composition comprising cells of a microorganism capable of expressing both the TPH and TDC enzymes is able to produce and secrete 5-HT in the mammalian gut (figure 2A). In contrast, a composition comprising cells of a microorganism capable of expressing the TDC enzyme is able to produce and secrete TRM in the mammalian gut (figures 1, 5B). The 5HT productivity of the microorganism can be modulated by the chosen combination of TPH and TDC enzymes expressed in the cells (Example 1). Accordingly, in some microorganisms the combination of expressed enzymes are (a) tryptophan 5-hydroxylase whose amino acid sequence has at least 80% sequence identity to SEQ ID No.: 6 or 12, and wherein the amino acid sequence of said tryptophan decarboxylase has at least 80% sequence identity to SEQ ID No.: 18.

In a further aspect, the microorganism in said composition comprises a recombinant nucleic acid molecule or transgene encoding tryptamine 5-hydroxylase, which catalyzes the conversion of O2 + reduced [NADPH—hemoprotein reductase] + tryptamine => H+ + H2O + oxidized [NADPH—hemoprotein reductase] + serotonin (figure 1). The assay and quantification of tryptamine yield is described in 1.1.2 and 1.1.3. Preferably, the microorganism in said composition further comprises a nucleic acid sequence encoding TDC. Following oral administration to a mammal, the microorganism in said composition, is capable of expressing the TPH and T5H enzymes in the mammalian gut such as to produce and secrete 5-HT (figure 1).

In a further aspect, the microorganism in said composition comprises nucleic acid molecules or transgenes encoding either TPH and TDC, or TDC and T5H, as well as the enzymes:
- serotonin acetyltransferase (AANAT) EC 2.3.1.87, which catalyzes the conversion of acetyl-CoA and serotonin to CoA and N- acetyl-serotonin and
- acetylserotonin O-methyltransferase (ASMT) (EC 2.1.1.4), catalyzes the last reaction in the production of melatonin from L-tryptophan, the conversion of N-acetyl-serotonin and S-adenosyl-L-methionine (SAM) to melatonin and S-adenosyl-L- homocysteine (SAH). SAH can then be recycled back to SAM via the S-adenosyl-L-methionine cycle in microbial cells where the S-adenosyl-L-methionine cycle is native (or exogenously added) and constitutively expressed, such as, e.g., in *E.coli* (figure 1).

In a further aspect, the microorganism is genetically modified by the introduction of heterologous nucleic acid molecules encoding said 5-HT or melatonin pathway enzymes, including TPH, TDC, T5H, AANAT and ASMT enzymes; where the heterologous nucleic acid molecules are each cognately linked to a promoter, such as a constitutive or inducible promoter. The heterologous nucleic acid molecules encoding said enzymes may be cloned within an operon linked to a common cognate promoter. The heterologous nucleic acid molecules may be cloned into a self-replicating episome introduced into the microorganism, or may be cloned into the chromosome of the microorganism. The episome may be a native plasmid of the microorganism or a heterologous plasmid. Preferably, said plasmid is devoid of genetic elements that facilitate transduction to another microbial cell; or alternatively the plasmid comprises genes encoding proteins essential for survival of said microbial cell (i.e. essential gene as described in Example 3).

In a further aspect thereof, the microorganism is further genetically modified, in being devoid of genes capable of expressing a functional *Trp* operon repressor protein, and/or a functional tryptophanase (EC:4.1.99.1) (figure 1). A microorganism that is unable to express a functional *Trp* operon repressor protein, lacks the repressor protein needed to

form a complex with L-tryptophan and bind the operator region of the trp operon (for example 5'-ACTAGT-'3') and is thus unable to prevent the initiation of transcription of the tryptophan biosynthetic pathway. A microorganism that is unable to express a functional tryptophanase (EC 4.1.99.1) is unable to catalyze the reaction: L-tryptophan + H(2)O <=> indole + pyruvate + NH(3). A microorganism of the invention devoid of genes capable of expressing a functional *Trp* operon repressor protein as well as a functional tryptophanase (EC 4.1.99.1) produces enhanced levels of 5-HT and TRM (Examples 2, 4 and figures 3, 6A) due to enhanced flux into the tryptophan pathway.

In a further aspect thereof, the microorganism is further genetically modified, to comprise a gene encoding a mutant GTP cyclohydrolase I EC 3.5.4.16 (GCHI), wherein the mutant provides for an increased hydroxylation activity of said TPH, by at least 2-fold, or 3-fold, or 4-fold, or 5-fold, 6-fold or, 7-fold or, 8-fold or, 9-fold or, 10-fold, as compared to a native non-mutant GCH1 (e.g. parent GCH1), as measured by 5HTP yield. GCH1 catalyzes the regeneration of the tetrahydrobiopterin cofactor from GTP, required by TPH synthesis (figure 1). A microorganism of the invention expressing a mutant GCH1 enzyme, that is devoid of both *trpR* and *tnaA* genes, produces increased amounts of 5-HT and TRM (Example 4, figure 5 A, 6A) due to enhanced flux towards 5HTP synthesis.

In a further aspect thereof, the microorganism comprises recombinant nucleic acid sequences encoding (a) tryptophan 5-hydroxylase (EC 1.14.16.4) and (b) tryptophan decarboxylase (EC:4.1.1.28), and is further genetically modified to regulate the relative expression levels of the TPH and TDC enzymes of the 5-HT pathway, such as to further increase the amounts of 5-HT produced, while reducing TRM production. By way of example, the expression of TPH and TDC is independently regulated by means of the RBS functionally linked to each of their respective nucleic acid coding sequences (i.e. genes). 5-HT yields are increased and TRM levels reduced, when the RBS of the TPH gene has a strength that exceeds the RBS of the TDC gene (measured as the relative strength of translation initiation), as shown in Example 4 (figure 5C, D; Figure 6A). The 5-HT productivity of a microorganism of the invention, devoid of *trpR* and *tnaA* genes, is synergistically increased when the translation rate of TPH gene transcript exceeds that of the TDC gene transcript, by virtue of their respective functionally linked RBS (figure 6A, B).

The composition according to the present invention is for use as a medicament for animals, in particular mammals selected from the group consisting of humans, dogs, cats, pigs, cattle, horses, goats, and sheep, as well as poultry (e.g. chickens). In a one embodiment, the composition is for use as a medicament for pregnant or lactating women.

In therapeutic applications, such as treating TRM; 5-HTP-, or 5-HT-related disorders of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI) and metabolism or melatonin-related disorders in a mammal, the composition according to the invention is for administration in an amount sufficient to at least partially cure or arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "a therapeutically effective dose". Amounts effective for this purpose will depend on a number of factors known to those skilled in the art such as the severity of the disease and the weight and general state of the patient.

In prophylactic applications, similarly, the composition according to the invention is for administration to a subject, susceptible to or otherwise at risk of a particular disease, in an amount that is sufficient to at least partially reduce the risk of developing a disease. Such an amount is defined to be "a prophylactic effective dose". Again, the precise amounts depend on a number of patient specific factors such as a patient's state of health and weight.

The microorganism of the invention can be provided as a pure form or can be incorporated in a matrix. Such matrix can advantageously protect the microorganism during the passage through the gastrointestinal tract (including the acidic conditions of the stomach) and enable live cells of the microorganism to arrive to the gut. Such protective matrix can comprise sugar(s) (such as maltodextrin), proteins or fat component. In one embodiment the protective matrix comprises or is a vegetable oil. The microorganism may be cultured according to any suitable method and prepared for encapsulation or addition to a nutritional composition by freeze-drying or spray-drying for example. Alternatively, it may be purchased already prepared in a suitable form for addition to food products.

A suitable nutritional composition includes a dairy product, a beverage powder, a dehydrated soup, a dietary supplement, a meal replacement, a nutritional bar, a cereal, a confectionery product, animal feed supplement or a dry pet food.

The composition of the present invention may further contain protective hydrocolloids (such as gums, proteins, modified starches), binders, film forming agents, encapsulating agents/materials, wall/shell materials, matrix compounds, coatings, emulsifiers, surface active agents, solubilizing agents (oils, fats, waxes, lecithins etc.), adsorbents, carriers, fillers, co-compounds, dispersing agents, wetting agents, processing aids (solvents) , flowing agents, taste masking agents, weighting agents, jellifying agents, gel forming agents, antioxidants and antimicrobials. The composition may also contain conventional pharmaceutical additives and adjuvants, excipients and diluents, including, but not limited to, water, gelatine of any origin, vegetable gums, ligninsulfonate, talc, sugars, starch, gum arabic, vegetable oils, polyalkylene glycols, flavouring agents,

preservatives, stabilizers, emulsifying agents, buffers, lubricants, colorants, wetting agents, fillers, and the like. In all cases, such further components will be selected having regard to their suitability for the intended recipient.

In a preferred aspect of the present invention, the composition further contains at least one prebiotic. Prebiotics can thus promote colonization of microorganism of the invention, in the intestines of a subject following oral administration and thereby enhance the effect of the microorganism contained in the composition according to the invention. Furthermore, several prebiotics have a positive influence on, e.g., digestion.

Preferably the prebiotic may be selected from the group consisting of oligosaccharides and optionally contain fructose, galactose, mannose, soy and/or inulin; dietary fibers; or mixtures thereof. The composition of the present invention may be provided in powder form having a water activity less than 0.2, for example in the range of 0.19-0.05, preferably less than 0.15.

Following oral administration, the microorganism of the invention is capable of surviving and/or colonizing one or more regions of the gut, including the duodenum, small intestine, cecum, proximal and distal colon. As exemplified in Example 6, the orally administered microorganism colonized primarily the cecum, proximal and distal colon.

Following colonization of the gut, the microorganism of the invention is shown to produce 5-HT, detectable in feces thereof, and importantly, to be accompanied by increased levels of 5-HT in the mucosal layers of the gut (Example 6). Furthermore, the colonizing microorganism is seen to induce therapeutically beneficial changes in both gene expression and gut physiology. In particular, observed changes in gut physiology include an increased mucosal layer, the maintenance of levels of markers of gut barrier function, and decreased levels of markers for gut inflammation, each of which is commensurate with a therapeutic effect on 5-HT related GI disorders. The expression of the 5-HT related receptors, HTR1B and HTR1D, in the gut was also observed, following administration of a microorganism of the invention producing elevated levels of 5-HT or TRM commensurate with a therapeutic effect on 5-HT related CNS disorders, including migraines (Example 8).

Administration of a composition according to the invention to a subject is demonstrated to have a prophylactic and therapeutic effect on a CNS related disorder from the pre-clinical trials preformed herein, using the well-established methods: Forced Swim Test (FST) and the Open Field Test (OFT) (Example 9 and 10).

II. In a second aspect, the invention provides a recombinant bacterium, comprising one or more recombinant nucleic acid molecules or transgenes encoding one of more

proteins selected from:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),

(b) tryptophan decarboxylase (EC 4.1.1.28), and

(c) tryptamine 5-hydroxylase (EC:1.14.-.-)

wherein said microorganism is devoid of genes capable of expressing *trp* operon repressor protein and tryptophanase (EC:4.1.99.1), and

wherein the cell is capable of producing increased amounts of one or more of 5-hydroxytryptophan, 5-hydroxytryptamine and tryptamine as compared to a cell of the non-recombinant microorganism from which it was derived.

In a further aspect thereof, said one or more recombinant nucleic acid molecules or transgenes encode proteins selected from the group:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),

(b) tryptophan decarboxylase (EC 4.1.1.28),

(c) tryptamine 5-hydroxylase (EC:1.14.-.-),

(d) tryptophan 5-hydroxylase (EC 1.14.16.4) and tryptophan decarboxylase (EC 4.1.1.28), and

(e) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-).

In a further aspect said bacterium comprises recombinant nucleic acid molecules encoding:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or

(b) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-), and

additionally recombinant nucleic acid molecules encoding:

(c) serotonin acetyltransferase (AANAT) EC 2.3.1.87 and

(d) acetylserotonin O-methyltransferase (ASMT) (EC 2.1.1.4),

wherein the cells are capable of producing melatonin, in amounts that are increased as compared to cells of the non-recombinant bacterium from which it was derived.

In a further aspect thereof, the bacterium further comprises a gene encoding a mutant GTP cyclohydrolase I (GCHI), whose amino acid sequence has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 % sequence identity to a wild-type GCHI having SEQ ID NO: 2, and comprises one or more mutations, wherein the mutant provides for an increased hydroxylation activity of said TPH, as compared to a native GCH1.

By way of example, the mutant GTP cyclohydrolase I (GCH1) is one having at least one or more mutations in an amino acid residue selected from the group consisting of D97, M99, T101, V102, A125, K129, N170, V179, T196, T198, S199, L200, S207, H212, E213, F214, L215 and H221, wherein the mutation in N170 is N170K, N170D or N170L; the mutation in V179 is V179A; the mutation in H212 is H212R or H212K; and the mutation in H221 is H221R or H221K. Preferably the mutation is a substitution selected from T198I, T198S, F214S, V179A, M99I and L200P.

The skilled person would be able to test for further mutants by co-expression of the mutant GCH1 and tryptophan 5-hydroxylase in a microbial cell and testing for increased activity of tryptophan hydroxylation to 5-hydroxytryptophan. Preferably, the mutant GCH1 increases the hydroxylation activity of tryptophan 5-hydroxylase by at least 2-fold, or 3-fold, or 4-fold, or 5-fold, 6-fold or, 7-fold or, 8-fold or, 9-fold or, 10-fold, as compared to the non-mutant GCH1 from which the mutant was derived, as measured by 5-hydroxytryptophan yield.

In a further aspect thereof, said bacterium is capable of expressing a protein having tryptophan 5-hydroxylase (EC 1.14.16.4) activity, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 6, 8, or 12.

In a further aspect thereof, said bacterium is capable of expressing a protein having tryptophan decarboxylase (EC 4.1.1.28) activity, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 14 or 18.

In a further aspect thereof, said bacterium is capable of expressing a protein having tryptamine 5-hydroxylase (EC:1.14.-.-) activity, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 20.

In a further aspect thereof, said bacterium is capable of expressing a protein having serotonin acetyltransferase EC 2.3.1.87 activity, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 22.

In a further aspect thereof, said bacterium is capable of expressing a protein having acetylserotonin O-methyltransferase (EC 2.1.1.4),activity, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 24.

In a further aspect thereof, said bacterium comprising recombinant nucleic acid molecules encoding (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and (b) tryptophan decarboxylase (EC 4.1.1.28), wherein the tryptophan 5-hydroxylase has at least 80% sequence identity to SEQ ID No.: 6 or 12, and wherein the amino acid sequence of said tryptophan decarboxylase has at least 80% sequence identity to SEQ ID No.: 18.

In a further aspect thereof, said bacterium comprising recombinant nucleic acid molecules encoding (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and (b) tryptophan decarboxylase (EC 4.1.1.28), is further genetically modified to up-regulate the expression level of TPH relative to TDC enzymes in the 5-HT pathway, such as to further increase the amounts of 5-HT produced, and reduce TRM production. By way of example, the nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are each functionally linked to their respective RBS, where the ratio of their strengths is at least 1.5: 1.0; 1.75: 1.0; 2.0:1.0; 2.5:1.0; 3.0:1.0; 3.5:1.0; 4.0:1.0; 4.5:1.0; 5.0:1.0; 5.5:1.0; 6.0:1.0; 6.5:1.0; 7.0:1.0; 7.5:1.0; 8.0:1.0; 8.5:1.0; 9.0:1.0; 9.5:1.0; 10.0:1.0; 20:1; 40: 1; 60: 1.0; 80: 1.0; 100:1.0; 150:1.0; 200:1.0; 250:1.0; and 300:1.0 more preferably at least 20.0:1.0; where RBS strength is measured as defined above, with reference to Salis at al., 2009.

Alternatively, said bacterium comprising recombinant nucleic acid molecules encoding (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and (b) tryptophan decarboxylase (EC 4.1.1.28), is further genetically modified to up-regulate the expression level of TDC relative to TPH enzymes in the 5-HT pathway, such as to further increase the amounts of TRM production. For this purpose, the nucleic acid molecules encoding tryptophan decarboxylase and tryptophan 5-hydroxylase are each functionally linked to their respective RBS, where the ratio of their strengths is at least 1.5: 1.0; 1.75: 1.0; 2.0:1.0; 2.5:1.0; 3.0:1.0; 3.5:1.0; 4.0:1.0; 4.5:1.0; 5.0:1.0; 5.5:1.0; 6.0:1.0; 6.5:1.0; 7.0:1.0; 7.5:1.0; 8.0:1.0; 8.5:1.0; 9.0:1.0; 9.5:1.0; 10.0:1.0; 20:1; 40: 1; 60: 1.0; 80: 1.0; 100:1.0; 150:1.0; 200:1.0; 250:1.0; and 300:1.0 more preferably at least 2.0:1.0;

In a further aspect thereof, one or more recombinant nucleic acid molecules encoding (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and/or (b) tryptophan decarboxylase (EC 4.1.1.28), in the bacterium of the invention, is functionally linked to a promoter that regulates expression constitutively or is inducible. When the promoter is a constitutive

promoter, a suitable promoter may be selected from among the synthetic promoters described in http://parts.igem.org/Promoters/Catalog/Anderson), preferably selecting a strong promoter having a measured strength of ≥0. 5 on the Anderson scale.

In a further aspect thereof, said bacterium is not capable of expressing a functional trp operon repressor protein, where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 26.

In a further aspect thereof, said bacterium is not capable of expressing a protein having tryptophanase activity (EC:4.1.99.1), where the amino acid sequence of said protein has at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO: 29.

In a further aspect thereof, said bacterium is not antibiotic resistant to one or more clinically-used antibiotic agents.

The recombinant bacterium according to the second aspect, in its various described forms, is suitable for use in a composition according to each of the further aspects of the invention.

EXAMPLES

Example 1: Genetically modified *Escherichia coli* cells express a biosynthetic pathway for 5-HT production

A 5-HT biosynthetic pathway was introduced into cells of *E. coli* Nissle to establish a synthetic serotonin pathway to catalyze the two consecutive metabolic steps of converting TRP to 5-HTP and then 5-HTP to 5-HT (Figure 1). Identification of the optimal combination enzymes to catalyze these two metabolic steps was determined by expressing and determining the 5-HT yield obtainable from the combination of TPH and TDC genes.

1.1 Methodology

1.1.1 Modified E. coli cells were engineered as follows, with reference to Table 1:

The host strain, *E. coli* Nissle 1917 (Mutaflor®) was purchased from Ardeypharm GmbH, Germany. The gene for green fluorescent protein (GFP, GenBank: CAH64882.1) was placed under control of a strong, constitutive promoter (Part BBa_J23101, Registry of Standard Biological Parts, www.parts.igem.org) and integrated into the *E. coli* Nissle

genome at the Tn7 attachment site using pGRG25 as described by McKenzie et al., (2006). Genes encoding TPH proteins derived from mammalian or plant sources: Human TPH1 (H1); human THP2 (H2); mouse TPH1 (M1); mouse THP2 (M2), and genes encoding TDC were derived from rice TDC (R), *Cataranthus roseus* (C) , guinea pig (G) and *Candidatus Koribacter versatilis* Ellin345 (ck) were synthesized employing codons optimized for *E. coli* using the online tool from IDT (idtdna.com/CodonOpt). The tested TPH and TDC gene combinations were cloned as an operon, under the control of a synthetic promoter BBa-J23107, in a self-replicating plasmid (using pUC as backbone), comprising a kanamycin resistance gene. The plasmids were subsequently transformed into the host strain, by employing standard cloning and transformation procedures known in the art.

1.1.2 Growth and in vitro metabolite production was determined as follows:

Strains were grown in a modified M9 medium, containing 1x M9 salts (M6030, Sigma Aldrich), 0.2% (w/v) glucose, 0.1% (w/v) casamino acids (Cat.No. C2000, Teknova), 1 mM MgSO4, 50 µM FeCl3, 0.2% (v/v) 2YT medium (composition see below), and 50 mg/L of L-tryptophan unless stated otherwise. Kanamycin was added at a final concentration of 50 mg/L unless stated otherwise. Three single colonies were picked from each strain, grown in 300 µl medium in 96 deep-well plates and shaken at 250 rpm at 37°C for 16 hours. The main culture was inoculated by diluting this pre-culture 1:100 into fresh medium, and cells were grown for 24 h under the same conditions. In the case of substrate feeding, TRP and 5-HTP was added to the growth medium in an amount of 100mg TRP or 5-HTP/L. Afterwards, the culture supernatant was separated from cells using a 0.2 µm pore size filter, and frozen at -20°C until analysis by LC-MS. All data shown are mean +/- SD from at least 3 biological replicates.

1.1.3 Metabolite production was quantified by LC-MS as follows:

Detection of serotonin, tryptamine, tryptophan and 5-HTP were conducted by liquid chromatography mass spectrometry (LC-MS) measurements on a Dionex UltiMate 3000 UHPLC (Fisher Scientific, San Jose, CA) connected to an Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). The system used an Agilent Zorbax Eclipse Plus C18 2.1 x 100 mm, 1.8 µm column kept at 35°C. The flow rate was 0.350 mL/min with 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) as mobile phase. The gradient started as 5% B and followed a linear gradient to 35% B over 1.5 min. This solvent composition was held for 3.5 min after which it was changed immediately to 95% B and held for 1 min. Finally, the gradient was changed to 5% B until 6 min. The sample (1 uL) was passed on to the MS equipped with a heated electrospray ionization source (HESI) in positive-ion mode with sheath gas set to 60 (a.u.), aux gas to 20 (a.u.) and sweep gas to 2 (a.u.). The cone and probe temperature

were 380°C and 380°C, respectively, and spray voltage was 3500 V. Scan range was 50 to 500 Da and time between scans was 100 ms. Detection of serotonin (160.07646 ion), tryptamine (144.08158 and 161.10775 ions) and tryptophan (205.09785 ion) was conducted in full scan whereas the 5-HTP detection was carried out after HCD fragmentation (221.09>162.05553, 25% HCD CE). Quantification of the compounds was based on calculations from calibration standards analyzed before and after sets of 24 samples. All reagents used were of analytical grade.

1.2 Results:

Genetically modified strains of the host, *E. coli* Nissle, engineered to express the mouse TPH1 (M1) or human TPH2 (H2), in combination with TDC from rice (R) or *Cataranthus roseus* (C) were found to produce measurable amounts of serotonin production (Figure 2A). When the strains were fed with the substrate 5-HTP during cultivation, cells expressing a gene encoding the rice TDC (R) produced the highest yields of 5-HT (79.5±11.1% yield), demonstrating that rice TDC was the most efficient in catalyzing the 5-HTP decarboxylation step (Figure 2B). Accordingly, *E. coli* Nissle, expressing the 'M1R' and 'H2R' pathways, being 5-HT producers were selected for further optimization.

Feeding to TDC showed that all enzymes produced some serotonin, but the 'R' enzyme from rice had the highest conversion (right panel).

Example 2. 5-HT and TRM production by genetically modified *Escherichia coli* cells of the invention is enhanced by increasing tryptophan pathway flux

Increased availability of tryptophan pathway substrate, tryptophan, is shown to increase 5-HT production in 5-HT producing strain of the invention (Figure 3). increase In light of this observation, the endogenous pools of available TRP were increased in genetically engineered cells of the invention were generated by inactivating endogenous genes encoding the tryptophan repressor trpR and tryptophanase tnaA, such as to enhance tryptophan pathway flux.

2.1 Methodology:

Knockouts of *trpR* and *tnaA* from the host *E. coli* Nissle genome were performed using CRISPR/Cas9 as described in (Mehrer et al., 2018). Briefly, a two-plasmid system consisting of an inducible cas9/λ-Red expression plasmid and a guide RNA (gRNA) plasmid were used to introduce double-strand breaks at the desired gene loci in the host genome. gRNAs were designed using CRISPy-web (Blin et al., 2016) after uploading the EcN genome sequence (GenBank: CP007799.1). Templates for homologous recombination at the selected cut site were generated as follows: where available, strains with the desired knockouts (ΔtrpR::FRT-kan-FRT or ΔtnaA::FRT-kan-FRT,

respectively) from the KEIO collection (Baba et al., 2006) were transformed with pSIJ8 (Jensen et al., 2015) and the FLP recombinase gene was induced to remove the kanamycin resistance gene. Resulting colonies were screened for kanamycin sensitivity. The ΔtrpR::FRT or ΔtnaA::FRT loci were amplified using oligos binding 500 bp up and downstream of the FRT site to generate PCR products of approximately 1 kb. CRISPR/Cas9 and gRNA expression plasmids were cured from the strains as described previously (Mehrer et al., 2018).

Growth of host cells; their metabolite production and quantification was performed as described in example 1.1.2 and 1.1.3.

2.2 Results:

In order to direct a corresponding flux of endogenous tryptophan towards the 5-HT pathway, the strains of the invention were further engineered by knockouts of genes encoding trpR and tnaA from the host genome to generate host *E. coli* Nissle strains designated NΔ2. An exogenous supply of tryptophan is shown to further enhance 5HT and TRM production by such genetically modified *E. coli* Nissle cells of the invention (strain EcNΔ2 + pUC-H2R in figure 3).

Example 3. Genetically modified *Escherichia coli* strains of the invention exhibit stable 5-HT production

When the 5-HT operon, encoding the 5-HT biosynthetic pathway, is cloned into a multi-copy plasmid in host cells of the invention, the production of 5-HT is dependent on the stability of the plasmids. Stability is preferably not dependent on plasmid genes conferring antibiotic resistance. Two solutions for conferring plasmid stability were compared; based on the plasmid backbone of pUC (as in example 1 and 2) and one of the two native plasmids in *E. coli* Nissle, pMUT1 (Blum-Oehler et al., 2003).

3.1 Methodology:

The high copy number 5-HT production plasmid (pUC-H2R) was modified by introducing a synthetic copy of the essential bacterial gene *infA* down stream of H2R pathway operon. The corresponding infA gene in the host genome was knocked out using the protocol described in Example 2.1, so as to generate *E.coli* Nissle strain NΔ3 (Δ*trp*R, Δ*tna*A, Δ*inf*A).

The operon comprising the H2R pathway was also cloned into plasmid pMUT1 (GenBank: A84793.1) as follows. The pMUT1 plasmid was isolated from wild-type E. coli Nissle, and amplified to serve as the pMUT backbone. Elements that could enable plasmid transfer were removed to generate pMUT (Δ bp 1 – 1323 and 1664 – 3117 from circular sequence

A84793.1), and a kanamycin resistance gene and the H2R or M1R operons (without *infA*) were inserted into the backbone to generate pMUT-H2R or pMUT-M1R, respectively.

The following additional versions of the plasmids, pMUT-H2R or pMUT-M1R, were generated: the hok/sok toxin-antitoxin plasmid stability element (GenBank: MK134376, Region 58002-58601) was inserted into the pMUT-M1R backbone to generate the plasmid pMUT14-ser. The TPH gene was removed from pMUT14-ser to generate pMUT11-trm, and both TPH and TDC were removed to make pMUT09-ctrl.

Cell growth determination: Cells were grown as described in example 1.1.2, but wherein after inoculation of the main culture, 200 µl were transferred into a flat-bottom, clear 96-well microtiter plate; then sealed with a gas-permeable membrane (Z380059, Sigma Aldrich). Cultures were grown at 37°C with 700 rpm orbital shaking, and their growth was recorded on a BioTek™ ELx800 plate reader at OD_{630nm} every 10 minutes.

Plasmid stability determination: six single colonies each were picked from each tested strain were grown in 300 µl 2YT medium (containing 1.6 % (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) without kanamycin in 96 deep-well plates and shaken at 250 rpm at 37°C for 24 hours. The optical density (OD630) of the cultures was measured and cultures were diluted 1:1000 into fresh medium, and cells were plated on LB-Agar plates with and without kanamycin daily, corresponding to approximately 10 doublings every 24h. Colony forming units on LB Agar plates +/- kanamycin were counted daily. At time points t=0, 50 and 100 generations, cultures were diluted 1:100 into the modified M9 medium described above (without kanamycin) and serotonin production was measured as described.

3.2 Results:

While stable, the pUC based plasmid expressing both the H2R pathway and *infA* genes caused an undesired growth defect in both NΔ2 and NΔ3 host cells (EcN with ΔtrpR, ΔtnaA, ΔinfA), likely due to metabolic burden (Figure 4 A, B and C).

By contrast, the pMUT based plasmid (pMUT-H2R) was both stably maintained in NΔ2 host cells (Figure 4 A, B) and showed no detectable fitness defect for at least 100 generations, in the absence of selection pressure, in the form of antibiotic resistance (Figure 4 A, B, C). Additionally, 5-HT production by EcNΔ2 pMUT-H2R cells was stable over this period and comparable to initial pUC-H2R titers (Figure 4D).

The hok/sok toxin-antitoxin module was introduced into the pMUT-M1R plasmids, producing pMUT14-ser and pMUT11-trm (Table 1) to further ensure their stability *in vivo*.

Example 4. Optimization of 5-HT production by genetically modified *Escherichia coli* strains of the invention

Tryptamine (TRM) is a significant byproduct detected among the metabolites produced by the genetically engineered E. coli strains of the invention expressing a plant TDC enzyme (Figures 2A, 3). A genetic modification, designed to enhance flux towards 5HTP synthesis, was to increase the activity of GTP glycohydrolase (GCH1) activity and thereby enhance regeneration of the tetrahydrobiopterin cofactor, required by TPH synthesis. Further genetic modifications designed to fine-tune the relative expression strength of TPH and TDC were tested in order to further enhance 5-HT production.

4.1 Methodology

The coding sequence of the *E. coli* N *folE* gene was mutated by first amplifying the *folE* gene in 2 parts, and then introducing the T198I encoded mutation in the oligos used for the overlap-extension PCR. The resulting dsDNA fragments were purified and co-transformed with the gRNA plasmid to generate markerless gene mutations of the *folE* gene in the genome of cells of EcN.

RBS strength libraries were designed using the 'RBS calculator v1.1' (Salis et al., 2009) choosing library sizes of 4-6 variants for each of the co-expressed genes encoding TPH and TDC (M1R and H2R), ranging in theoretical strength from 50 to 50000 arbitrary units. Mutations in the RBS were introduced upstream of each gene with oligos containing degenerate bases and using Gibson assembly (Gibson et al., 2009). Transformants were picked randomly and RBSs sequenced after testing the strains' metabolite production profiles.

4.2 Results

The native *folE* gene in host *E. coli* Nissle cells of the invention was mutated to encode a mutant GCH1 (T198I) in order to enhance regeneration of the tetrahydrobiopterin cofactor. The production of 5-HT is increased, in these Optimized Nissle (oN) strains expressing the mutant GCH1 enzyme in combination with the 5-HT biosynthesis operon (oN pMUT1H2R or oN pMUTM1R) as compared to strains lacking this additional modification (NΔ2 pMUT-H2R or NΔ2 pMUT-M1R respectively) as seen in figure 5A. The mutant GCH1 increases the hydroxylation of tryptophan to 5-hydroxytryptophan catalyzed by human tryptophan 5-hydroxylase in strains comprising pMUT10-5htp, by 4-fold (figure 5F).

oN strains, expressing the mutant GCH1 enzyme in combination with either the 5-HTP biosynthesis pathway (oN10 = oN pMUTM1), or TRM pathway (oN11 = oN pMUTR)

produced 5-HTP and TRM as the only detected products of the pathway, respectively (Figure 5B).

Modulating the relative expression strength of TPH and TDC using selected combinations of ribosome binding sites (RBS) for their respective gene is shown to enhance 5-HT production and reduce accumulation of TRM (Figure 5C and D). Increased 5-HT production was obtained when the relative strength of the RBS functionally linked to the TPH gene exceeded that of the RBS functionally linked to the TDC gene.

The synergistic effect of the genetic modifications introduced into *E. coli* Nissle strains comprising genes of the 5-HT pathway, on their respective 5-HT productivity and 5-HT:TRM ratio is demonstrated in Figure 6. Knockout of the *trpR* and *tnaA* genes in the *E. coli* genome conferred a synergistic effect on 5-HT yields and 5-HT:TRM ratio in an *E. coli* cell provided that the relative expression of the TPH gene exceeded that of the TDC gene, by virtue of the functionally linked RBS (figure 6A). Introduction of the *fol*E genomic mutation (encoding a T198I substitution) further increased 5-HT yields and 5-HT:TRM ratio when combined with the foregoing mutations in the *E. coli* Nissle strains (figure 6B).

Example 5 Oral administration of a genetically modified *Escherichia coli* strain of the invention is predicted to produce therapeutic 5-HT levels in a subject

Therapeutically effective levels of 5-HT in the gut are reported to lie in the range of 5-30 mg/L (Liu, Q. et al., 2008). The relative abundance of 5-HT producing bacteria required to produce 5-HT within the therapeutically effective range in mouse gut is predicted for bacteria having an *in vivo* 5-HT production rate of 0.1 to 10 mg/L*OD_{630}*h (figure 6C). This is based on underlying assumptions with respect to density of bacteria in gut predicted to range from 1E2 - E11 /g (Sender et al., 2016), depending on the location (Casteleyn et al., 2010), as shown below.

Intestinal segment	Volume		Cell density*	
	cm^3	%	cells/g (ml)	cells total
Duodenum	0.21	15.7	100	21
Jejunum	0.69	51.5	1000	690
Ileum	0.07	5.2	50000000	3500000
Caecum	0.16	11.9	1E+10	1600000000
Colon–rectum	0.21	15.7	1E+11	21000000000
Sum	1.34	100		2.3E+10

* *E. coli*: 1 OD600nm (in standard 1cm gap length spectrophotometer) = 0.36 g/L = 8*E8 cells/ml

In vitro, specific productivity (P) can be measured as serotonin titer (in mg/L) per bacterial load (in OD_{630nm}) per hour. Since the 5-HT depletion rate is unknown, it is set to be equal to productivity in order to determine physiologically relevant concentrations (in mg/L). The genetically modified *E. coli* strains of the invention (e.g. strain oN14) when administered arepredicted to be capable of producing 5-HT *in vivo* at near physiologically relevant levels.

Example 6 Oral administration of a genetically modified *Escherichia coli* strain of the invention enhances 5-HT production in the mouse gut

Oral delivery of the genetically modified *E. coli* Nissle strains of the invention and their localization to the gut and enhancement of 5-HT production was demonstrated in mice.

6.1 Methodology

6.1.1 Oral administration: Male mice (C57BL/6) supplied by Taconic Biosciences or Jackson Laboratories, from 6-8 weeks of age were group-housed on a 12-h light:dark cycle (lights on from 7:00-19:00) at constant temperature with ad libitum access to food and water in a Specific Pathogen Free (SPF) facility. Upon delivery, mice were given one week to adjust to new location, followed by a 7-day, 3-rotation microbiome normalization protocol. On day one, three, and five of the normalization protocol, mice were randomly mixed. On day 6, mice were mixed one last time and separated in their respective cohorts with 2-3 cages per cohort with 3-5 mice in each cage, exact numbers depending on experiment. On day seven, mice began day zero of the experimental protocols, being tagged for identification and housed in groups of 3-5 mice in bottle fed cages, set in designated racks, and opened only under a laminar flow hood. Fecal samples were collected at day zero of the experimental protocol. The mice were then orally gavaged every 24hrs at the same time of day for 10 days with 200µl of either sterile PBS or freshly grown 10^9 cells of *E. coli* Nissle strains designated: oN14 (*E. coli* Nissle strain oN comprising plasmid pMUT14-ser); oN11 (*E. coli* Nissle strain oN comprising plasmid pMUT11-trm), and oN9 (*E. coli* Nissle strain oN comprising plasmid control plasmid pMUT9-ctrl), in sterile PBS (Figure 5E). 24 hours after the last gavage, fecal pellets were collected and mice were either euthanized for serum, intestinal tissue, and intraluminal fecal collection or were used for motility or behavioral testing.

6.1.2 Gut localization of oN strains: was determined by plating digesta derived from different intestinal regions on LB agar growth media comprising 50 mg/L kanamycin and quantifying the number of GFP positive, kanamycin resistant (KanR) colonies.

6.1.3 Immunodetection in thin sections: Colon samples were collected and 5mm sections were cut in the mid-colon and fixed in either a Methanol-Carnoy (60% Methanol, 30%

Chloroform, 10% Acetic Acid) Solution or 4% Paraformaldehyde (PFA) in Phosphate Buffered Solution (PBS) for 24 hours. Fixed samples were then washed three times and stored in 70% ethanol. Samples were kept intact with digesta in the lumen, embedded in Paraffin and cut luminally at 4 µm and either prepped for Immunohistochemistry or subjected to Hematoxylin and Eosin staining. For Immunohistochemistry: PFA fixed slides were used for Serotonin (5-HT) antibody staining and Methacarn fixed samples were used for Muc2, Green Fluorescent Protein (GFP), and Chromogranin A (CgA) antibody staining. All slides were baked for 2 hours at 57°C, deparaffinized in xylene and an ethanol-water gradient, rehydrated, then treated with an antigen retrieval solution of 10mM sodium citrate, pH 6.0 for 30 minutes at 90°C. Slides were blocked for 30 minutes at room temperature with 5% fetal bovine serum (FBS) in PBS and then incubated with primary antibodies at 4°C overnight in 5% FBS in PBS. Slides were washed with PBS three times, incubated with secondary antibody for 2 hours at room temperature in PBS, washed three times in PBS, incubated with DAPI (4',6-diamidino-2-phenylindole) at 10µg/ml for 5 minutes, washed three times in PBS and mounted using glycerol. Slides stained with primary antibodies pre-conjugated with Alexa fluorophores did not undergo secondary antibody staining. Antibodies Used: Anti-GFP rabbit IgG with Alexa Fluor 594 Conjugate (Life Technologies A21312). Anti-Muc2 polyclonal rabbit (Santa Cruz Technologies sc-15334). Anti-Chromagranin A polyclonal rabbit (Abcam ab15160). Anti-Serotonin polyclonal goat (Abcam ab66047). Secondary Anti-Rabbit Alexa Fluor 568 (Thermo-Scientific Novex a11011). Secondary anti-goat IgG Alexa Fluor 488 (Abcam ab150077). All images were taken with Nikon Eclipse Ti2-E Inverted Microscope System, image conversion and cell size (particle) analysis performed using NIS-Elements, Advanced Research Software and ImageJ (FIJI) for Macbook version 2.0.0-rc-69/1.52i.

6.1.4 *In vivo* metabolite analysis. Serum, tissue samples, and fecal samples were analyzed for serotonin, 5-hydroxytrytophan, tryptamine, and tryptophan. For serum: blood samples were collected via the inferior vena cava post euthanasia, samples were mixed 1:1 with 0.9% NaCl, 0.2% Ascorbic acid. Samples were kept at room temperature for 10 minutes then placed on ice until centrifugation at 2000g for 10 minutes at 4°C. Supernatant was saved for analysis and immediately frozen at -20°C. For tissue analysis: colon tissue was weighed and immediately frozen on liquid nitrogen after collection and cryofractured using stainless steel microvials (Biospec 2007) with a single 6.34 mm stainless steel bead (Biospec 11079635ss) beat for 30s in BeadBeater (Biospec 112011). Tissue was then resuspended in 0.9% NaCl, 0.1% Ascorbic Acid, centrifuged at 2000g for 10 minutes at 4°C. Supernatant was saved for analysis and immediately frozen at -20°C. For fecal samples: samples were weighed, placed in Corning Cryovials with 0.5 ml of 0.9% NaCl, 0.1% Ascorbic Acid with 200ml Zirconia/Silica beads (Biospec 11079101z)

and beat for 5 minutes in BeadBeater at 4°C. Samples were centrifuged at 2000g for 10 minutes at 4°C. Supernatant was saved for analysis and immediately frozen at -20°C.

6.2 Results

Following 10 days of oral administration of 10^9 cells of *E. coli* Nissle strains oN14, oN11 and control strain oN9 (Figure 5E) to mice, the administered cells were primarily found to accumulate in their large intestine. The highest concentration of oN cells was detected in the proximal colon with $>10^8$ CFU/g (Figure 7A), while fewest oN bacteria were detected in the small intestine with ($<10^4$ CFUs/g). In the mid-colon, the oN cells were localized along the mucosal layer based on their visualization by immuno-histochemistry with an anti-GFP antibody (Figure 7B).

The level of TRP, 5-HTP, 5-HT and TRM were also measured in samples of serum, colonic tissue, and feces derived from the orally-treated mice. Tryptophan levels were elevated in colon tissues and feces, but not serum, and only in mice orally administered *E. coli* Nissle strain oN14 (Figure 8A). 5-HT levels were not statistically different in serum and in colonic tissue across cohorts ($p>0.4$ by ANOVA). However, fecal 5-HT levels were significantly increased in mice administered *E. coli* Nissle strain oN14 (13.4-fold increase, $p<0.0001$ by ANOVA) compared to other groups (Figure 8B). Tryptamine and 5-HTP levels were below the limit of detection in all samples.

Although 5-HT levels were not elevated in colonic tissues *per se*, (following washing to remove residual digesta), increased levels of serotonin were specifically detected along the mucosal layers of the colon in mice administered *E. coli* Nissle strain oN14 (Figure 8C). These results demonstrate that orally administered cells of *E. coli* Nissle strain oN14 accumulate and produce 5-HT *in vivo* along the mucosa of the colon in mice.

Example 7 Oral administration of a genetically modified *Escherichia coli* strain of the invention elicits changes in gene expression and physiology in the gut

Oral administration of *E. coli* Nissle strain oN14 to mice was shown to upregulate Muc2 expression and increase mucosal thickening, which in turn confers increased mucosal integrity. Excess serotonin derived from enterochromaffin cells can stimulate intestinal inflammation. However, even though increased levels of 5-HT were detected in mucosal layers of the colon of treated mice (Example 6), this did not lead to a change in expression of markers of intestinal inflammation, intestinal turnover or intestinal barrier function (not shown).

Host (mouse) TPH2 expression, but not TPH1, was increased in the colon of mice administered *E. coli* Nissle strain oN14 indicating that bacterially-derived 5-HT can induce TPH2-mediated neuronal 5-HT biosynthesis in the ENS of mice. The total number

or size of EC cells (the primary producers of host 5-HT in the gut) in these mice was not however affected.

While tryptophan and serotonin metabolism can also affect the kynurenine pathway, whose dysfunction is linked to many neurologic and metabolic disorders, no changes were detected in the expression of genes encoding the rate-limiting enzymes that convert L-tryptophan to N-formylkynurenine, in the colon of mice administered *E. coli* Nissle strain oN14.

7.1 Methodology

7.1.1 Oral administration: as described in 6.1.1.

7.1.2 Gene expression analysis: Tissue cryofractured according to the protocol in Example 6.1.4. Tissue was then resuspended in Trizol (Invitrogen) according to the instructions of the manufacture for RNA isolation. Total RNA was resuspended in 30 µL of RNase-free water. RNA was converted to cDNA with iScript gDNA Clear cDNA synthesis Kit (BioRad 1725035) according to manufacture instructions. Quantitative Real Time polymerase chain reaction (qPCR) was performed in triplicates in 384 well-plates on Applied Biosystems QuantStudio6 Flex Real-Time PCR System according to manufacture instructions. Primers used are listed in Table 3. Cyclophilin was used as the housekeeping gene. Relative mRNA levels were quantified using the $\Delta\Delta C_T$ method.

7.1.3 Immunodetection in thin sections: as described in example 6.1.3.

7.2 Results

Expression of the MUC2 gene in colonic tissues derived from mice administered *E. coli* Nissle strain oN14 was increased ~2-fold ($p<0.05$ by ANOVA) compared to control groups (Figure 9A). Correspondingly, sections of colonic tissue immunohistochemically stained with anti-Muc2 antibodies, showed a more prominent mucosal layer in mice administered *E. coli* Nissle strain oN14 (Figure 9B). Hematoxylin and Eosin staining of the colonic sections further confirmed the lack of histopathological changes associated with gut inflammation (not shown).

No difference, however, was observed in expression of goblet cell markers CDX2 or intestinal stem cell markers BM1 and LGR5 (not shown), indicating that Muc2-producing goblet cells and general intestinal turnover are unchanged. Correspondingly, no changes in gene expression levels of the inflammation markers: IL6, IL1β, NFKβ, IL17A, and TNFα by qPCR were detected in colon tissues of mice administered *E. coli* Nissle strain oN14 (not shown). Furthermore, no changes in colon length ($p=0.66$ by ANOVA) were observed, this being another indicator of chronic intestinal inflammation if found to be

shortened (not shown). The expression of TJP1, TJP2, or OCLN, markers for maintenance of intestinal barrier in the colon, also remained unchanged in mice administered *E. coli* Nissle strain oN14 (not shown).

While no difference was observed in expression of TPH1 and SERT, an unexpected 2.8-fold increase in TPH2 expression was detected in mice administered *E. coli* Nissle strain oN14 compared to other groups ($p<0.001$ by ANOVA) in the colon (Figure 9A). However, to change in the total number or size of EC cells (the primary producers of host 5-HT in the gut) as quantified by qPCR and by immunohistochemistry of the EC cell marker chromogranin A was detected (not shown).

Additionally, no changes were detected in the expression of *IDO1, IDO2,* and *TDO2*, in mice administered *E. coli* Nissle strain oN14, which encode the rate-limiting enzymes that convert L-tryptophan to N-formylkynurenine (not shown).

Example 8 Oral administration of a genetically modified *Escherichia coli* strain of the invention enhances expression of serotonin receptors and associated effects in the gut

Serotonin is a ligand for a number of 5-HT receptors, which are GPCRs mediating excitatory and inhibitory neurotransmission throughout the ENS, CNS, and the peripheral nervous system. Ligand activation of the 5HT1b receptor in the CNS has been linked to reduced aggressiveness (de Almeida et al., 2002) and, along with 5HTr1d, it is the target receptor for tryptamine-based treatment of migraines (Tepper et al., 2002). Oral administration of both *E. coli* Nissle strain oN11 and oN14 to mice was shown to increase HTR1B and HTR1D gene expression, suggesting a possible tryptamine-mediated effect.

The 5HT3 receptor has a wide variety of physiological roles, implicated in emesis, irritable bowel syndrome (IBS), schizophrenia, anxiety, learning, memory and addiction, and is modulated clinically by antiemetic 5HTr3 antagonists (Thompson et al., 2007). The 5HT4 receptor induces neurogenesis in the enteric nervous system, and is linked to modulation of peristalsis in the GI tract, stress-induced feeding behavior, and altered learning and memory, and depression in the CNS (Gershon et al., (2007); Lucas et al., (2007); Lamirault et al., (2001)). While 5HTr7 stimulation can improve cognition and memory (Meneses et al., 2015), and 5HTr7 antagonism can resolve antidepressive and anti-psychotic behaviors in the CNS (Roth, B.L. et al., 1994), its function in the gut is not well understood although it has been linked to IBS and inflammatory bowel disease (IBD) (Guseva, D. et al., 2014). In view of the diversity and magnitude of the detected changes in gene expression of 5-HT receptors it is plausible that E.coli strain oN14 elicits physiological responses in the host.

Upregulated expression of HTR3, HTR4 and HTR7 genes was detected in the oN14 cohort only, indicating a specific serotonin-mediated response.

Additionally oral administration of *E. coli* Nissle strain oN14 is shown to decrease total GI transit, consistent with serotonin being known to modulate GI motility by inducing both intraluminal pressure to cause peristaltic reflex and the colonic migrating motor complexes that sweep through the intestine during intervals of fasting.

Although changes in gut motility and bacterially-produced biogenic amines might affect the gut microbiome, no significant change in the gut microbiome were detected between mice cohorts as a result of administration of *E. coli* Nissle strains oN14, oN11, oN9 or PBS alone, based on a 16S metagenomic sequencing of their fecal matter (not shown). Together, administration of *E. coli* Nissle strains oN14 and oN11 elicits a number of physiological responses in the gut, including host 5-HT receptor gene expression and GI motility, while leaving the resident microbial community undisturbed.

8.1 Methodology

8.1.1 Oral administration: as described in 6.1.1.

8.1.2 Gene expression analysis: as described in 7.1.1.

8.1.3 Total gastrointestinal transit: Carmine red, which cannot be absorbed from the lumen of the gut, was used to study total GI transit time. A 6% solution of carmine red (300 µl; Sigma-Aldrich) suspended in 0.5% methylcellulose (Sigma-Aldrich) was administered by gavage through a 21-gauge round-tip feeding needle. The time at which gavage took place was recorded as T0. After gavage, fecal pellets were monitored at 10-minute intervals for the presence of carmine red. Total GI transit time was considered as the interval between T0 and the time of first observance of carmine red in stool.

8.1.4 Metagenomic 16S sequencing and analysis: Fecal pellets were collected from mice at Day 0 and Day 10 and immediately stored at -20□C until paired-end sequencing. Genomic DNA was extracted from fecal pellets using the Epicenter gram positive kit plus an initial bead beating step with 0.1mm Zirconia beads. PCR amplification of the 16S rRNA V4 region and multiplexed barcoding of samples were done in accordance with previous protocols57. The V4 region of the 16S rRNA gene was amplified with 1x NEBNext q5 Hot Start HiFi PCR Master Mix using custom primers according to the method from Kozich et al., (2013). Sequencing was done with the Illumina MiSeq system (300V2 kit). Sequenced pair reads were prepared using USEARCH v10.0.240_i86osx64. Forward and reverse reads were paired and filtered to a minimum length of 240 with maximum expected error of 1. Sequences were dereplicated, clustered with minimum cluster size of 2, and mapped to OTUs at 97% identity58. OTU taxonomy was assigned with the RDP

classifier59. Sequences aligned using PyNAST within Conda with QIIME1, python 2.7, matplotlib 1.4.3. Tree built with FastTree read with RStudio package ape. OTU data analysis and visualization preformed using Graphpad Prism and RStudio with gglot2 and phyloseq packages. Statistical significance performed by ANISOM using RStudio with phyloseq and vegan packages.

8.2 Results

An increase in expression of HTR1B and HTR1D genes was detected in mice administered *E. coli* strains oN11 and oN14 (both are $p<0.05$ by ANOVA) (Figure 10 a, b), while increased expression of HTR3, HTR4 and HTR7 genes was only observed in colons of mice administered *E. coli* strain oN14 (all are $p<0.01$ by ANOVA) (Figure 10 c - e).

Furthermore, administration of *E. coli* strain oN14 specifically decreased total GI transit time by as much as 15% ($p<0.05$ by ANOVA) in treated mice as compared to control cohorts (Figure 10). 16S metagenomic sequencing on fecal matter derived from mice cohorts following administration of *E. coli* Nissle strains oN14, oN11, oN9 or PBS alone, at Day 0 and Day 11 revealed no significant change in the gut microbiome between cohorts from Day 0 to Day 11 (R: 0.07403, significance: 0.155, by ANISOM) (not shown).

Example 9 Oral administration of a genetically modified Escherichia coli strain of the invention reduced anxiety in mice

Oral administration of genetically modified *E. coli* oN strains of the invention are shown to induce behavioral changes in treated mice, that may be mediated through the gut-brain axis or through an increase in peripheral and/or brain 5-HT levels. Two well-established methods, the Forced Swim Test (FST) and the Open Field Test (OFT) were employed to demonstrate these behavioral changes. The FST, commonly used to assess efficacy of anxiolytics and antidepressants in rodents, quantifies behavioral despair by recording the time spent immobile in a water filled container as a measure of hopelessness to escape the stressful environment. The OFT measures anxiety levels and willingness to explore in a stressful environment by detecting total fecal boli, total distance traveled, and time spent exploring the inner and outer zones of a 50x50cm white, well-lit, open field.

Results of these tests demonstrate that oral administration of *E. coli* strain oN14 to mice has a therapeutic effect on anxiety-related disorders.

9.1 Methodology

9.1.1 Oral administration: as described in 6.1.1

9.1.2 Forced Swim Test: Each mouse was placed in clear cylindrical tanks (30cm height x 20cm) with 15cm of water at 23-25°C for 6 minutes and recorded, as described by Ferguson (2001). Mice were then towel dried and placed under a heat lamp to dry to prevent hypothermia and then placed back in their original cage. Recordings were de-identified and a blinded observer, scored recordings for the time spent immobile during the last 4 minutes of the 6-minute test, as described by Can et al., (2012).

9.1.3 Open Field Test: Each mouse was placed in a white, open-roof, well lite, 50 cm (length) x 50 cm (width) x 38 cm (height) chamber and recorded for 10 minutes following previously described methods (Spohn, S.N. et al., 2016). Mice were then placed back into their original cage. Fecal pellets produced during the 10-minute test were counted. Recordings were then post processed and analyzed using BehaviorCloud as previously described (Spohn, S.N. et al., 2016). Mouse tracking software recorded total path of movement of each mouse, total distance traveled, time spent in the inner zone defined as the inner 40 cm square and outer zone defined as the outer 10 cm border closest to the walls, number of times entering the inner zone, and distance traveled within the inner zone.

9.2 Results

The FST and OFT was performed on mice cohorts following administration of *E. coli* Nissle strains oN14, oN11, oN9 or PBS alone.

In the FST, mice cohorts administered *E. coli* Nissle strain oN14 exhibited a statistically reduced immobility time during FST by up to 17% ($p=0.004$ by ANOVA) as compared to the control cohorts administered E. coli strain oN (Figure 11a). This result was robustly replicated in a separate and independent animal cohort (not shown).

In the OFT, mice cohorts administered *E. coli* Nissle strain oN14 showed a decreased number of fecal boli during the ten-minute test (Figure 11b), which could be associated with reduced stress or the decreased total GI transit (Figure 10f). While wild-type mice naturally prefer closed, dark, tight spaces, mice cohorts administered *E. coli* Nissle strain oN14 spent an increased amount of time exploring the inner 40x40cm zone and decreased time spent near the edges along the 10x10cm outer zone (figure 11d, e), with no change in total distance traveled (Figure 11c). These observed effects in the OFT were confirmed by an OFT on another cohort of mice (not shown). Additional OFT on mice obtained from a different commercial vendor (Jackson Laboratory) having different initial gut microbiota also gave similar physiological and behavioral results as above (not shown).

Example 10 Efficacy of oral administration of a genetically modified Escherichia coli strain of the invention matches SSRIs in mice

Fluoxetine, sold under the brand names Prozac and Sarafem among others, is a commonly used antidepressant of the selective serotonin reuptake inhibitor (SSRI) class. It is used to treat e.g. major depressive disorder, post-traumatic stress syndrome (PTSD), and other indications. The efficacy of oral administration of genetically modified *E. coli* oN strains of the invention is shown to correspond to administration of fluoxetine, by the ability to induce behavioral changes in treated mice indicative of a therapeutic reduction in anxiety or stress.

10.1 Methodology:

10.1.1 Administration: as described for oral administration in 6.1.1, but with the following modifications: The mice were administered sterile PBS only (PBS) or freshly-grown 10^9 cells of *E. coli* Nissle strain designated oN14 (*E. coli* Nissle strain oN comprising pMUT14-ser) in sterile PBS (oN14) by oral gavage. Additionally, the mice were administered a 200μl intraperitoneal (I.P.) injection of saline or the SSRI, fluoxetine at 10mg/kg body weight in saline. The mice received the oral and IP administered co-treatments every 24hrs at the same time of day for 21 days. Accordingly, the four mice treatment groups received: IP: saline/Gavage: PBS (negative control); IP: Fluoxetine/Gavage: PBS (SSRI positive comparator), IP: saline/Gavage: oN14 (oN14 positive comparator); and IP: Fluoxetine/Gavage: oN14 (positive combination comparator). 24 hours after the last administration, the mice were subjected to motility and behavioral testing.

10.2 Results

The phenotypes exhibited by mice after 21 days treatment with oN14 (IP: saline/Gavage: oN14) closely resemble those treated with Fluoxetine (IP: Fluoxetine/Gavage). As seen in figure 12, A) A reduction in fecal boli produced during the OFT is observed both with oN14, Fluoxetine or the combination therapy (IP: Fluoxetine/Gavage: oN14), indicating a reduction in stress level leading to less defecation. Further, in figure 12 B) - E) the time spent and distance travelled in the inner zone, as well as the total number of entries in the inner zone, are all increased by either oN14 or fluoxetine treatment. An increase of these parameters is interpreted as a reduction in anxiety or stress, and demonstrates an increased willingness to explore in an unknown environment. In the Forced Swim Test (figure 12 F), the time spent not moving is measured as an indication of hopelessness / depression. oN14 and Fluoxetine treatment both significantly reduce the time of immobility, indicating similar efficacy. oN14 treated mice showed similar effects on time spent in the inner zone, entries in the

inner zone, and distance in the inner zone as compared to mice treated with Fluoxetine (Figure 12). The combination of treatment with oN14 and treatment with fluoxetine yielded a small reduction in the total effect when compared to the individual groups treated with only oN14 or fluoxetine.

Example 11 Oral administration of a genetically modified *Escherichia coli* strain of the invention increases plasma and urine serotonin

Oral administration of the 5-HTP-producing *E. coli* Nissle strain oN10 to mice was shown to increase plasma serotonin and urine serotonin and 5-HTP concentrations in mice.

11.1 Methodology

11.1.1 Oral administration: as described in 6.1.1. with the following modifications: Animals received Streptomycin (5 g/L) in the drinking water from 3 days before gavage and throughout the experiment. A single oral gavage of 108 cells of either oN10 or a control EcN strain without tryptophan hydroxylase (EcN_Control). Animals were treated with the TDC inhibitor Carbidopa via intraperitoneal injection (IP) every 24h, and a fresh fecal sample was collected daily for 7 days, after which the animals were euthanized. Plasma samples were taken on day 2 and day 7 after gavage.

11.1.2 *In vivo* metabolite analysis: Plasma, tissue, gut content, urine and fecal samples were analyzed for serotonin, 5-hydroxytrytophan, tryptamine, 5-Hydroxyindoleacetic acid (5-HIAA) and tryptophan. For plasma: blood samples were collected via the submandibular vein on day 2 (and by heart puncture day 8) post gavage, kept on ice for 10 min in Li-Heparin Microtainers, and then plasma was separated by centrifugation at 10000g at 4°C for 90 sec. and plasma snap-frozen at -80C. An internal standard buffer containing 0.9% NaCl, 0.2% Ascorbic acid, and 20 mg/L C-13-labelled tryptophan was added after thawing the samples, and proteins were precipitated using methanol extraction. After drying samples using a vacuum centrifuge, they were reconstituted in 50ul ddH_2O for LC-MS/MS analysis. For tissue analysis: colon tissue was weighed and immediately frozen on liquid nitrogen after collection and cryofractured using stainless steel microvials (Biospec 2007) with a single 6.34 mm stainless steel bead (Biospec 11079635ss) beat for 30s in BeadBeater (Biospec 112011). Urine was collected on day 2 and day 6 post gavage, and immediately snap-frozen at -80°C. An internal standard buffer containing 0.9% NaCl, 0.2% Ascorbic acid, and 20 mg/L C-13-labelled tryptophan was added after thawing the samples, and proteins were precipitated using methanol extraction. After drying samples using a vacuum centrifuge, they were reconstituted in 50ul ddH_2O for LC-MS/MS analysis.

11.2 Results

A significant increase in 5-HT concentration was observed in the plasma with oN10 compared to a control strain without TPH expression ($p<0.001$ by two-tailed t-test), demonstrating successful oN10-derived production of 5-HTP *in vivo* (figure 13 A). The amount of 5-HTP produced by oN10 was sufficient to elevate peripheral plasma serotonin concentrations while leaving tryptophan concentrations unaffected (figure 13 B), indicating a specific effect of oN10 on serotonin metabolism within physiologically relevant levels. Plasma concentrations of 5-HTP were below quantification levels in all samples; however, since 5-HTP is known to cross the blood-brain-barrier, peripherally produced 5-HTP is able to increase serotonin biosynthesis in the brain, with potential therapeutic effects on mood, sleep, anxiety and other disorders (Turner et al., 2006). The oN10 strain presents a mechanism to deliver 5-HTP from within the GI tract at a constant dosage, circumventing undesirable effects of dosage-dependent fluctuations in neurotransmitters like 5-HTP and 5-HT.

Urine 5-HT and 5-HTP concentrations were also increased with oN10 compared to the control group (figure 13 C and D, respectively), showing increased serotonergic metabolism with oN10.

Example 12: Genetically modified *Saccharomyces cerevisiae* cells express a biosynthetic pathway for 5-HT production

Saccharomyces cerevisiae (*S. cerevisiae*) was genetically modified by the introduction of recombinant genes to establish a synthetic serotonin pathway comprising enzymes to catalyze the two consecutive metabolic steps of converting TRP to 5-HTP and then 5-HTP to 5-HT (Figure 1). Identification of the optimal combination of enzymes to catalyze these two metabolic steps was determined by expressing and determining the 5-HT yield obtainable from the combination of TPH and TDC genes.

12.1 Methodology

Modified *S. cerevisiae* cells were engineered as follows, with reference to Table 1:

The host strain, *S. cerevisiae* was obtained from Mans et al., (2015). Genes encoding TPH proteins derived from mammalian, plant or bacterial sources: mouse TPH1 (M1) and rice TDC (R) were the same as in 1.1.1. A different TDC gene from the bacterium *Candidatus Koribacter versatilis* Ellin345 (ck) was also tested. The pathway genes were cloned into either the yeast 2μ plasmid backbone, or the ARS/CEN backbone (see figure 14). The M1 gene was cloned under control of the PKG1 promoter, and the TDC genes (either rice TDC or ck TDC) were controlled by the TEF1 promoter. The plasmids were subsequently transformed into the host strain, by employing standard cloning and transformation procedures known in the art.

Growth and *in vitro* metabolite production was determined as follows: Yeast strains were grown in Synthetic Complete Dropout (SC) medium (Yeast Nitrogen Base Without Amino Acids 6.7 g/L, glucose 20 g/L, drop-out mix 2 g/L). The drop-out mix used lacked histidine, thereby maintaining the plasmids. The negative control (containing no plasmids) was grown with 20mg/L histidine. Three single colonies were picked from each strain, grown in 2ml µl medium in 24 deep-well plates and shaken at 250 rpm at 30°C for 48 hours. The main culture was inoculated to OD_{600} = 0.05 in 25 ml SC medium + 500mg/L tryptophan in shake flasks, and cells were grown for 72h under the same conditions. Afterwards, the culture supernatant was separated from cells by centrifugation at 17000 x g for 2 minutes, and frozen at -20°C until analysis by HPLC. All data shown are mean +/- SD from at least 3 biological replicates.

Metabolite production was quantified by LC-MS as in 1.1.3

12.2 Results:

Genetically modified strains of the host, *S. cerevisiae*, engineered to express the mouse TPH1 (M1), in combination with TDC from rice (R) [or a bacterial TDC gene from *Candidatus Koribacter versatilis* Ellin345 (ck_TDC) produced significant amounts of serotonin (Figure 15 A). While in the control strain without TPH or TDC gene expression (Sc_Control), no serotonin production is detected ($p<0.0018$, ANOVA), strains expressing mouse TPH1 and R-TDC (pSc-1 and pSc-2) or ck-TDC (pSc-3) (see table 1) produced circa 0.38 +/- 0.07 mg/L and 0.52 +/- 0.06 mg/L serotonin, respectively. The ck-TDC gene can also functionally replace the R-TDC gene and enables production of serotonin in *E. coli* constructs (figure 15 B), but strong overexpression of ck-TDC causes cellular toxicity in oN (figure 15 C) leading to a reduction in growth rate and final biomass yield compared to R-TDC expression.

Example 13: Genetically modified *Bacillus subtilis* 168 cells express a biosynthetic pathway for 5-HT production

Bacillus subtilis (*B. subtilis*) was genetically modified by the introduction of recombinant genes to establish a synthetic serotonin pathway comprising enzymes to catalyze the two consecutive metabolic steps of converting TRP to 5-HTP and then 5-HTP to 5-HT (Figure 1). Identification of the optimal combination enzymes to catalyze these two metabolic steps is determined by expressing and determining the 5-HT yield obtainable from the combination of TPH and TDC genes.

13.1 Methodology

The host strain, *B. subtilis* 168, obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) [NC_000964], was genetically engineered as follows,

with reference to Table 1:Genes encoding TPH proteins derived from mammalian or plant sources: mouse TPH1 (M1) and rice TDC (R) were the same as in 1.1.1. The tested TPH and TDC gene combination, together with the T198I variant of the *E. coli* folE gene, were cloned into an integrative plasmid (derived from pDG364, see Table 1) to facilitate genomic integration of the pathway operon. The genes were placed in a synthetic operon consisting of a strong, *Bacillus*-specific promoter (Ps11), a strong RBS (R01) for M1, a medium-strength RBS for TDC (RBS 50 from Table 1), and a strong RBS (R11) for folE(T198I). Plasmid pBs-M1Rf contained the mouse TPH1, rice TDC, and folE(T198I) genes (Figure 16).

Cloning was performed in *E. coli* Top10 by employing standard molecular biology methods. The plasmids were subsequently transformed into the host strain, by employing standard cloning and transformation procedures known in the art. Correct pathway integration into the *B. subtilis* 168 genome at the *amyE* gene locus was verified by Sanger sequencing.

Growth and in vitro metabolite production was determined as follows: *B. subtilis* 168 strains were grown in Lysogeny Broth (LB) medium containing 0.2% (w/v) glucose and 50 mg/L of L-tryptophan. Three single colonies were picked from each strain, grown in 300 µl medium in 96 deep-well plates and shaken at 250 rpm at 37°C for 16 hours. The main culture was inoculated by diluting this pre-culture 1:100 into fresh medium, and cells were grown for 24 h under the same conditions. Afterwards, the culture supernatant was separated from cells using a 0.2 µm pore size filter, and frozen at -20°C until analysis by LC-MS. All data shown are mean +/- SD from at least 3 biological replicates.

Metabolite production was quantified by LC-MS as in 1.1.3.

13.2 Results:

Genetically modified strains of the host, *B. subtilis* 168, engineered to express the mouse TPH1 (M1), in combination with TDC from rice (R) and *E. coli* folE(T198I) are expected to produce of serotonin and 5-HTP.

No 5-HTP or 5-HT is produced using the wild-type *Bacillus subtilis* 168 strain, while expression of tryptophan hydroxylase M1, together with folE(T198I), will enable 5-HTP production. Expression of M1_TPH, folE(T198I) and R_TDC is expected to result in production of serotonin.

Table 1: Strains, plasmids and genes used in the examples

Strains*		
Name	**Genetic features**	**Source**
N	Wild-type *E. coli* Nissle 1917 (EcN)	Ardeypharm GmbH
NΔ2	EcN Δ*trpR*, Δ*tnaA*, *attTn7*::*GFP*	this study
NΔ3	EcN Δ*trpR*, Δ*tnaA*, Δ*infA*, *attTn7*::*GFP*	this study
NΔ2 –pMUT1	EcN Δ*trpR*, Δ*tnaA*, *attTn7*::*GFP* , cured of pMUT1	this study
oN	EcN Δ*trpR*, Δ*tnaA*, *folE*(T198I), *attTn7*::*GFP* , cured of pMUT1	this study
oN9	oN + pMUT09-ctrl	this study
oN10	oN + pMUTM10-5htp	this study
oN11	oN + pMUT11-trm	this study
oN14	oN + pMUT14-ser	this study
Sc	*Saccharomyces cerevisiae* strain derived from CEN.PK2-1C. Genotype: MATa; his3D1; leu2-3_112; ura3-52; trp1-289	Mans, R. et al. 2015
Bs	Wild-type *Bacillus subtilis* 168	DSMZ
EcN_Control	EcN_ Tn7::GFP, folE(T198I), ΔtyrR, StrepR + pMh-empty	this study
*Sequences for the Δ*trpR* (SEQ ID NO.:27), Δ*tnaA* (SEQ ID NO.:30), Δ*infA* (SEQ ID NO.:33), *folE*(T198I) (SEQ ID NO.: 3), *and attTn7*::*GFP* (SEQ ID NO.:34) can be found in the sequence list.		
Plasmids **		
Name	**Genetic features**	**Source**
pMUT1	Native plasmid of E. coli Nissle 1917	Genbank A84793.1 (SEQ ID NO.: 35)
pMUT	nucleotides 1 – 1323 and 1664 – 3117 from pMUT1	this study
pUC-H2R	P_{BBa_J23107}, human cTPH2 (RBS=5000), rice TDC (RBS=10000), *infA*, pUC backbone	GeneArt® / this study
pUC-H2R$_{opt}$	P_{BBa_J23107}, human cTPH2 (RBS=5000), rice TDC (RBS=700), *infA*, pUC backbone	this study
pUC-H2R$_{D}$	P_{BBa_J23107}, human cTPH2 (RBS=5000), rice TDC (RBS=50), *infA*, pUC backbone	this study

pUC-H1R	P_{BBa_J23107}, human cTPH1 (RBS=5000), rice TDC (RBS=10000), *infA*, pUC backbone	this study
pUC-M1R	P_{BBa_J23107}, mouse cTPH1 (RBS=3700), rice TDC (RBS=10000), *infA*, pUC backbone	this study
pUC-M2R	P_{BBa_J23107}, mouse cTPH2 (RBS=5000), rice TDC (RBS=10000), *infA,* pUC backbone	this study
pUC-H2G	P_{BBa_J23107}, human cTPH2 (RBS=5000), guinea pig TDC (RBS=10000), infA, pUC backbone	this study
pUC-H2C	P_{BBa_J23107}, human cTPH2 (RBS=5000), *Catharanthus roseus* TDC (RBS=10000), infA, pUC backbone	this study
pMUT-H2R	P_{BBa_J23107}, human cTPH2 (RBS=5000), rice TDC (RBS=700), pMUT1 backbone	this study
pMUT-M1R	P_{BBa_J23107}, mouse cTPH1 (RBS=2300), rice TDC (RBS=250), pMUT1 backbone	this study
pMUT09-ctrl	P_{BBa_J23101}, pMUT1 backbone, *hok/sok* locus	this study
pMUT10-5htp	P_{BBa_J23107}, human cTPH2 (RBS=10000), pMUT1 backbone, *hok/sok* locus	this study
pMUT11-trm	P_{BBa_J23101}, rice TDC (RBS=700), pMUT1 backbone, *hok/sok* locus	this study
pMUT14-ser	P_{BBa_J23101}, mouse THP1 (RBS=18000), rice TDC (RBS=700), pMUT1 backbone, *hok/sok* locus	this study
pDG364	*B. subtilis* integrative plasmid for insertion into the genomic *amyE* locus	Hoa, N. T. et al (2002)
pBs-M1Rf	P_{PS1}, mouse TPH1 (RBS = R0*), rice TDC (RBS = 50), folE(T198I) (RBS = R1*), backbone from pDG364	this study * Guiziou, S. *et al.* (2016)
pARS/CEN	pRS413U	NEB
p2u	pESC-HIS3-ccdB-USER	Agilent Catalog #217451
pSc_1	P_{TEF1}, rice TDC, P_{PKG1}, mouse TPH1, ARS/CEN backbone	this study
pSc_2	P_{TEF1}, rice TDC, P_{PKG1}, mouse TPH1, 2u backbone	this study
pSc_3	P_{TEF1}, ck TDC, P_{PKG1}, mouse TPH1, ARS/CEN backbone	this study
** Sequences for promoters P_{BBa_J23101} and P_{BBa_J23107} are found in the sequence list: SEQ ID NO.: 36 and 37.		
Genes/Proteins		

Wt *folE* (GCHI)	GTP cyclohydrolase I (SEQ ID NO.: 2)	Locus tag ECOLIN_12170, Protein AID79293.1
infA	translation initiation factor IF-1 (SEQ ID NO.: 32), encoded by native gene on E.coli Nissle genome (SEQ ID NO.: 31)	Locus tag ECOLIN_04590, Protein AID77906.1
hok/sok genes	toxin-antitoxin plasmid stability element	GenBank: MK134376, bp 58002-58601
H1 = hTPH1	TPH1 from *Homo sapiens*, catalytic domain: amino acids 103 to 416	UniProt ID P17752 (SEQ ID NO.: 9, 10)
H2 = hTPH2	TPH2 from *Homo sapiens*, catalytic domain: amino acids 144 to 462	UniProt ID Q8IWU9 (SEQ ID NO.: 11, 12)
M1 = mTPH1	TPH1 from *Mus musculus*, catalytic domain: amino acids 106 to 419	UniProt ID P17532 (SEQ ID NO.: 5, 6)
M2 = mTPH2	TPH2 from *Mus musculus*, catalytic domain: amino acids 142 to 460	UniProt ID Q8CGV2 (SEQ ID NO.: 7, 8)
R = rTDC	TDC from *Oryza sativa subsp. japonica* (Rice)	Uniprot ID Q6ZJK7 (SEQ ID NO.: 17, 18)
G = gTDC	TDC (called AADC, aromatic-amino-acid decarboxylase in mammals) from *Cavia porcellus* (Guinea pig)	UniProt ID P22781 (SEQ ID NO.: 15, 16)
C = cTDC	TDC from *Catharanthus roseus* (Madagascar periwinkle)	UniProt ID P17770 (SEQ ID NO.: 13, 14)
ck = ckTDC	TDC from *Candidatus Koribacter versatilis* Ellin345	Sequence ID: ABF41161.1 (SEQ ID No.: 110, 111)
***Bacillus subtilis* promoter and RBS sequences**		
P_PS1 (promoter)	actgcgtcaatacacgttgacactcttttgAAAGTGtgttaaattatcag	SEQ ID No.: 105
R0 (RBS)	GATTAACTAATAAGGAGGACAAAA	SEQ ID No.: 106
R1 (RBS)	GCTCTTAAGGAGGATTTTAGA	SEQ ID No.: 107

***S. cerevisiae* promoter sequences**		
P_TEF1		SEQ ID No.: 108
P_PKG1		SEQ ID No.: 109

Table 2. RBS variants used in the examples

mouse TPH1 RBS variants			
RBS ID in text/figures	**RBS Sequence 5'-3'**	**Translation Initiation Rate (au)**	**SEQ ID NO.**
M1 RBS=63000	AGGTATTATGCTAGCAGCTCATCAATACCCC CCCAATAAGGAGGATTAAG	63141	38
M1 RBS=35000	AGGTATTATGCTAGCAGCTCATCAATACCCC CCCAACAAGGAGGATTAAG	35174	39
M1 RBS=18000	AGCTCATCAATACCCCCCCAATAAGGAGGAT TAAG	18000	40
M1 RBS=13000	AGGTATTATGCTAGCAGCTCATCAATACCCC CCCAATCAGGAGGATTAAG	13670	41
M1 RBS=4200	AGGTATTATGCTAGCAGCTCATCAATACCCC CCCAACCAGGAGGATTAAG	4242	42
M1 RBS=2300	AGCTCATCAATACCCCCCCAAAAATAAGGAT TAAG	2300	43
human TPH2 RBS variants			
RBS ID in text/figures	**RBS Sequence 5'-3'**	**Translation Initiation Rate (au)**	**SEQ ID NO.**
H2 RBS=36000	AGCTCATCAATACCCCCCCATAAGCGAGGTT TAAG	36793	44
H2 RBS=10000	AGCTCATCAATACCCCCCCATAACCGAGGTT TAAG	10818	45
H2 RBS=5200	AGCTCATCAATACCCCCCCATAAGCAAGGTT TAAG	5265	46
H2 RBS=5000	AGCTCATCAATACCCCCCCAAAAATAAGGAT TAAG	5000	47
H2 RBS=3000	AGCTCATCAATACCCCCCCATAACCAAGGTT TAAG	2933	48

rice TDC RBS variants			
RBS ID in text/figures	**RBS Sequence 5'-3'**	**Translation Initiation Rate (au)**	**SEQ ID NO.**
R RBS=10000	GAAGAAATTATCAGAGAGAGGGAAGGTAACAC	10000	49
R RBS=3800	TACCCGTAGCTAAGAAGAAATTATCAGAGAGAGGGTAGGTAACAC	3877	50
R RBS=1800	TACCCGTAGCTAAGAAGAAATTATCAGAGAGACGGTAGGTAACAC	1887	51
R RBS=700	TACCCGTAGCTAAGAAGAAATTATCAGAGAGAGGGTCGGTAACAC	733	52
R RBS=250	AAGCTAAGAAGAAATTATCAGAGGGTCGGTAACAC	250	53
R RBS=50	TACCCGTAGCTAAGAAGAAATTATCAGAGAGACGGTCGGTAACAC	49	54

Table 3: qPCR primers used in the examples

	Forward Primer:		**Reverse Primer:**	
Gene	**Sequence**	**SEQ ID NO.**	**Sequence**	**SEQ ID NO.**
CYCLO	5'-GACGAAGGTAGCCAGTCACAAG-3'	55	5'-AATCAGGCCTGTGGAATGTGAG-3'	56
MUC2	5'-ATGCCCACCTCCTCAAAGAC-3'	57	5'- GTAGTTTCCGTTGGAACAGTGAA-3'	58
CDX2	5'-CAAGGACGTGAGCATGTATCC-3'	59	5'-GTAACCACCGTAGTCCGGGTA-3'	60
BM1	5'-AAATCCCCACTTAATGTGTGTCC-3'	61	5'- CTTGCTGGTCTCCAAGTAACG-3'	62
LGR5	5'-GGACCAGATGCGATACCGC-3'	63	5'- CAGAGGCGATGTAGGAGACTG-3'	64
IL6	5'-CTGCAAGAGACTTCCATCCAG-3'	65	5'-AGTGGTATAGACAGGTCTGTTGG-3'	66
IL1β	5'- GCAACTGTTCCTGAACTCAACT-3'	67	5'-ATCTTTTGGGGTCCGTCAACT-3'	68
NFKβ	5'-ATGGCAGACGATGATCCCTAC-3'	69	5'-TGTTGACAGTGGTATTTCTGGTG-3'	70
IL17A	5'-TTTAACTCCCTTGGCGCAAAA-3'	71	5'- CTTTCCCTCCGCATTGACAC-3'	72
TNFα	5'- CAGGCGGTGCCTATGTCTC-3'	73	5'-CGATCACCCCGAAGTTCAGTAG-3'	74
TPH1	5'- AACAAAGACCATTCCTCCGAAAG-3'	75	5'- TGTAACAGGCTCACATGATTCTC-3'	76
TPH2	5'-GTGACCCTGAATCCGCCTG-3'	77	5'-GGTGCCGTACATGAGGACT-3'	78

SERT	5′- CTCCGCAGTTCCCAGTACAAG-3′	79	5′- CACGGCATAGCCAATGACAGA-3′	80
CHGA	5′- CAGGCTACAAAGCGATCCAG-3′	81	5′- GCCTCTGTCTTTCCATCTCC-3′	82
TJP1	5′-GCCGCTAAGAGCACAGCAA-3′	83	5′-TCCCCACTCTGAAAATGAGGA-3′	84
TJP2	5′- GTTTGCCGTTCAGCAGCTTAG-3′	85	5′-CTTCAAAACCTCGGTCGTCAT-3′	86
OCLN	5′-TGAAAGTCCACCTCCTTACAGA-3′	87	5′-CCGGATAAAAAGAGTACGCTGG-3′	88
IDO1	5′-CAAAGCAATCCCCACTGTATCC-3′	89	5′-ACAAAGTCACGCATCCTCTTAAA-3′	90
IDO2	5′-CCTCATCCCTCCTTCCTTTC-3′	91	5′-GGAGCAATTGCCTGGTATGT-3′	92
TDO2	5′-AGGAACATGCTCAAGGTGATAGC-3′	93	5′-CTGTAGACTCTGGAAGCCTGAT-3′	94
HTR1b	5′-CGCCGACGGCTACATTTAC-3′	95	5′-TAGCTTCCGGGTCCGATACA-3′	96
HTR1d	5′-ATCACCGATGCCCTGGAGTA-3′	97	5′-GCGAGAAGAGTGGAGGGATG-3′	98
HTR3	5′-CCTGGCTAACTACAAGAAGGGG-3′	99	5′-TGCAGAAACTCATCAGTCCAGTA-3′	100
HTR4	5′-AGTTCCAACGAGGGTTTCAGG-3′	101	5′-CAGCAGGTTGCCCAAGATG-3′	102
HTR7	5′-TGCGGGGAGCAGATCAACTA-3′	103	5′-GACAAAGCACACCGAGATCAC-3′	104

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Claims

1. A composition for use as a medicament, wherein said composition comprises cells of a recombinant microorganism, and wherein said microorganism comprises one or more recombinant nucleic acid molecules encoding one or more proteins selected from:
   (a) tryptophan 5-hydroxylase (EC 1.14.16.4)
   (b) tryptophan decarboxylase (EC 4.1.1.28) and
   (c) tryptamine 5-hydroxylase (EC:1.14.-.-).

2. The composition for use according to claim 1, wherein said microorganism is devoid of genes capable of expressing:
   i. Trp operon repressor protein, and/or
   ii. Tryptophanase (EC 4.1.99.1).

3. The composition for use according to claim 1, wherein said microorganism comprises recombinant nucleic acid molecules encoding :
   (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or
   (b) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-), and

   further comprises recombinant nucleic acid molecules encoding:
   (c) serotonin acetyltransferase (EC 2.3.1.87) and
   (d) acetylserotonin O-methyltransferase (EC 2.1.1.4),

   wherein the cells are capable of producing melatonin.

4. The composition for use according to any one of claims 1 to 3, wherein said microorganism comprises a nucleic acid molecule encoding a mutant GTP cyclohydrolase I (EC 3.5.4.16), wherein the mutant provides for an increased hydroxylation activity of said tryptophan 5-hydroxylase of at least 2-fold as compared to non-mutant GCH1, preferably a mutant having an amino acid sequence having at least about 80% sequence identity to SEQ ID No.: 2, and comprises a mutation selected from T198I, T198S, F214S, V179A, M99I and L200P.

5. The composition for use according to any one of claims 1 to 4, wherein said microorganism comprises nucleic acid molecules encoding:
   (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and

(b) tryptophan decarboxylase (EC 4.1.1.28),

wherein said nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively; wherein the translation initiation strength of said first ribosomal binding site is at least ten fold greater than said second ribosomal binding site.

6. The composition for use according to claim 1 or 2, wherein said microorganism comprises nucleic acid molecules encoding:

(a) tryptophan 5-hydroxylase (EC 1.14.16.4), and

(b) tryptophan decarboxylase (EC 4.1.1.28),

wherein said nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively; wherein the translation initiation strength of said first ribosomal binding site is at least two-fold lower than said second ribosomal binding site.

7. The composition for use according to any one of claims 1 to 6, wherein said microorganism is selected from among *Escherichia, Bacteroides, Clostridium, Feacalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Staphylococcus* and *Bacillus.*

8. The composition for use according to any one of claims 1 to 7, for use in preventing and/or treating TRM-; 5-HTP-; 5-HT-; or melatonin-related disorders of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI); hormonal imbalance, metabolic disease, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, diabetes in an animal.

9. The composition for use according to claim 8, wherein said disorder of the central nerve system is selected from group consisting of anxiety- and depression-related behavior, memory-, cognition-, and psychiatric-disorders; generalized anxiety disorder, phobia disorder, social anxiety disorder, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, chronic stress disorder, separation and situational anxiety, age-related memory decline, dementia and disorders in sleep, spatial memory formation, alertness, focus, learning, and cognition; autism and migraine; and wherein said disorder of the gastro intestine is selected from the group consisting of immune-associated and inflammation-associated sickness; inflammatory bowel

disease, irritable bowel syndrome (IBS); celiac disease, diverticular disease, and colorectal cancer.

10. The composition for use according to any one of claims 1 to 9, wherein said composition is for oral administration to a mammal in need thereof.

11. A recombinant bacterial cell comprising one or more recombinant nucleic acid molecules or transgenes encoding one of more proteins selected from:
    (a) tryptophan 5-hydroxylase (EC 1.14.16.4),
    (b) tryptophan decarboxylase (EC 4.1.1.28), and
    (c) tryptamine 5-hydroxylase (EC 1.14.-.-)
wherein said bacterium is devoid of genes capable of expressing:
    i. trp operon repressor protein and
    ii. tryptophanase (EC:4.1.99.1).

12. The recombinant bacterial cell according to claim 11, wherein the bacterial cell is selected from among *Escherichia, Bacteroides, Clostridium, Feacalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Staphylococcus* and *Bacillus*.

13. The recombinant bacterial cell according to claim 11 or 12, wherein the amino acid sequence of said tryptophan 5-hydroxylase has at least 80% sequence identity to SEQ ID No.: 6 or 12, and wherein the amino acid sequence of said tryptophan decarboxylase has at least 80% sequence identity to SEQ ID No.: 18.

14. The recombinant bacterial cell according to any one of claims 11 to 13, wherein said bacterium comprises nucleic acid molecules encoding:
    (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and
    (b) tryptophan decarboxylase (EC 4.1.1.28),
wherein said nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively;
wherein the translation initiation strength of said first ribosomal binding site is at least ten fold greater than said second ribosomal binding site.

15. The recombinant bacterial cell according to any one of claims 11 to 14, wherein said bacterium comprises nucleic acid molecules encoding:
    (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and
    (b) tryptophan decarboxylase (EC 4.1.1.28),
    wherein said nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively,
    wherein the translation initiation strength of said first ribosomal binding site is at least two-fold lower than said second ribosomal binding site.

16. The recombinant bacterial cell according to claim 11 comprising recombinant nucleic acid molecules encoding :
    (a) tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or
    (b) tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-), and
    further comprises recombinant nucleic acid molecules encoding:
    (c) serotonin acetyltransferase (EC 2.3.1.87) and
    (d) acetylserotonin O-methyltransferase (EC 2.1.1.4), wherein the cells are capable of producing melatonin.

17. The recombinant bacterial cell according to any one of claims 11-16, wherein said bacterium comprises a nucleic acid sequence encoding a mutant GTP cyclohydrolase I (EC 3.5.4.16), wherein the mutant provides for an increased hydroxylation activity of said tryptophan 5-hydroxylase of at least 2-fold as compared to non-mutant GCH1.

18. The recombinant bacterial cell according to claim 17, wherein the amino acid sequence of said mutant GTP cyclohydrolase I (EC 3.5.4.16) has least about 80% sequence identity to SEQ ID No.: 2, and comprises a substitution selected from T198I, T198S, F214S, V179A, M99I and L200P.

19. The recombinant bacterial cell according to any one of claims 11 to 18, wherein the bacterial cell is not antibiotic resistant to one or more clinically used antibiotic agents.

20. A method of treating and/or preventing a TRM-; 5-HTP-; 5-HT-; or melatonin-related disorder in a subject, the method comprising administering to the

subject diagnosed with a TRM-; 5-HTP-; 5-HT-; or melatonin-related disorder recombinant bacteria engineered to express one or more

(a) tryptophan 5-hydroxylase (EC 1.14.16.4),

(b) tryptophan decarboxylase (EC 4.1.1.28), , or

(c) tryptamine 5-hydroxylase (EC 1.14.-.-).

21. The method according to claim 20, wherein said microorganism is devoid of genes capable of expressing:

a. a protein that functions as a Trp operon repressor protein, and/or

b. tryptophanase (EC 4.1.99.1).

22. The method according to claim 20 or 21, wherein said microorganism comprises recombinant nucleic acid molecules encoding:

a. tryptophan 5-hydroxylase (EC 1.14.16.4), and tryptophan decarboxylase (EC 4.1.1.28), or

b. tryptophan decarboxylase (EC 4.1.1.28) and tryptamine 5-hydroxylase (EC:1.14.-.-), and further comprises recombinant nucleic acid molecules encoding:

c. serotonin acetyltransferase (EC 2.3.1.87), and

d. acetylserotonin O-methyltransferase (EC 2.1.1.4),

and wherein the cells are capable of producing melatonin.

23. The method according to any one of the claims 20 to 22, wherein said microorganism comprises a nucleic acid molecule encoding a mutant GTP cyclohydrolase I (EC 3.5.4.16), wherein the mutant provides for an increased hydroxylation activity of said tryptophan 5-hydroxylase of at least 2-fold as compared to non-mutant GCH1.

24. The method according to claim 23, wherein the mutant GTP cyclohydrolase I (EC 3.5.4.16), wherein the amino acid sequence of the mutant GTP cyclohydrolase I has at least 80% sequence identity to SEQ ID No.: 2, and comprises a mutation selected from T198I, T198S, F214S, V179A, M99I and L200P.

25. The method according to any one of claims 20 to 24, wherein said microorganism comprises nucleic acid molecules encoding:

a. tryptophan 5-hydroxylase (EC 1.14.16.4), and

b. tryptophan decarboxylase (EC 4.1.1.28),

wherein said nucleic acid molecules encoding tryptophan 5-

hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively; wherein the translation initiation strength of said first ribosomal binding site is at least ten fold greater than said second ribosomal binding site.

26. The method according to any one of claims 20 to 24, wherein said microorganism comprises nucleic acid molecules encoding:
    a. tryptophan 5-hydroxylase (EC 1.14.16.4), and
    b. tryptophan decarboxylase (EC 4.1.1.28),
    wherein said nucleic acid molecules encoding tryptophan 5-hydroxylase and tryptophan decarboxylase are functionally linked to a first ribosomal binding site and a second ribosomal binding site respectively; wherein the translation initiation strength of said first ribosomal binding site is at least two-fold lower than said second ribosomal binding site.

27. The method according to any one of claims 20 to 26, wherein said microorganism is selected from among *Escherichia, Bacteroides, Clostridium, Feacalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Lactococcus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Staphylococcus* and *Bacillus*.

28. The method according to any one of claims 20 to 27, wherein the subject is a human.

29. The method according to claim 28, wherein the TRM-; 5-HTP-; 5-HT-; or melatonin-related disorder is a disorder of the central nerve system (CNS); enteric nervous system (ENS); gastro intestine (GI); hormonal imbalance, metabolic disease, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, or diabetes.

30. The method according to claim 29, wherein the disorder of the central nerve system is selected from group consisting of anxiety- and depression-related behavior, memory-, cognition-, and psychiatric-disorders; generalized anxiety disorder, phobia disorder, social anxiety disorder, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, chronic stress disorder, separation and situational anxiety, age-related memory decline, dementia and

disorders in sleep, spatial memory formation, alertness, focus, learning, and cognition; autism and migraine; and wherein said disorder of the gastro intestine is selected from the group consisting of immune-associated and inflammation-associated sickness; inflammatory bowel disease, irritable bowel syndrome (IBS); celiac disease, diverticular disease, and colorectal cancer.

Figure 1

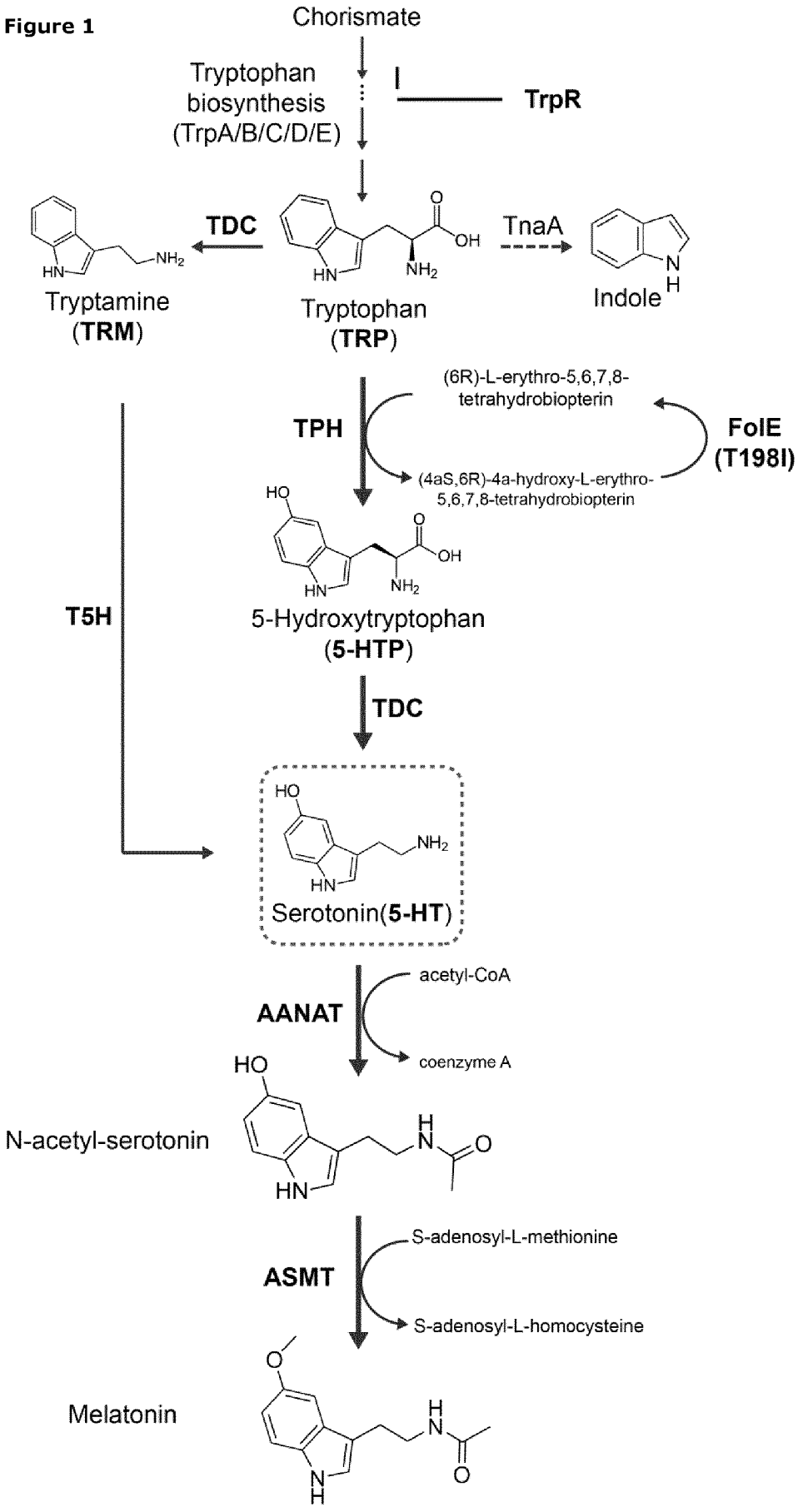

Chorismate
Tryptophan biosynthesis (TrpA/B/C/D/E)
TrpR
TDC
TnaA
Tryptamine (TRM)
Tryptophan (TRP)
Indole
TPH
(6R)-L-erythro-5,6,7,8-tetrahydrobiopterin
(4aS,6R)-4a-hydroxy-L-erythro-5,6,7,8-tetrahydrobiopterin
FolE (T198I)
T5H
5-Hydroxytryptophan (5-HTP)
TDC
Serotonin(5-HT)
AANAT
acetyl-CoA
coenzyme A
N-acetyl-serotonin
ASMT
S-adenosyl-L-methionine
S-adenosyl-L-homocysteine
Melatonin

Figure 2

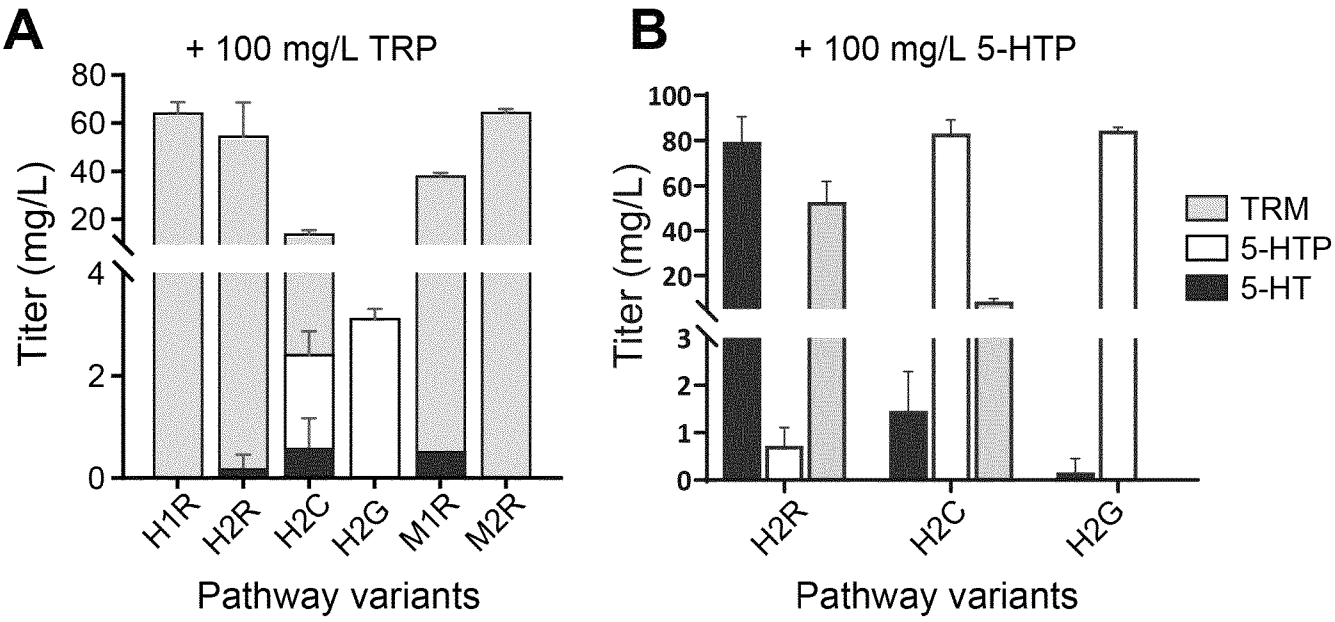
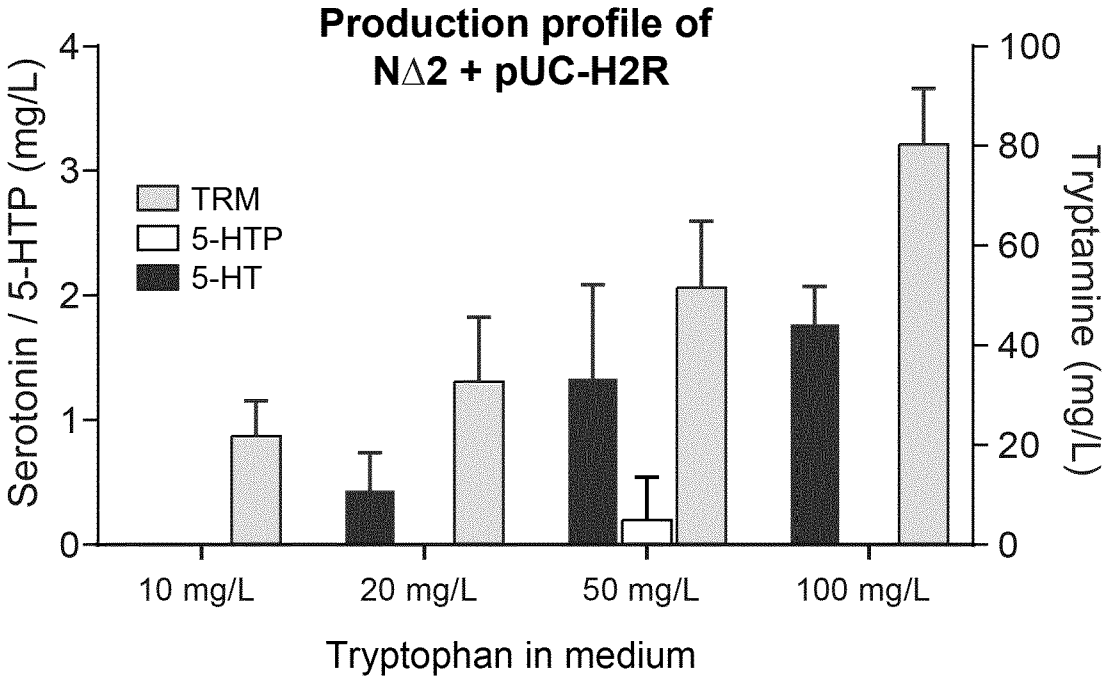


Figure 3



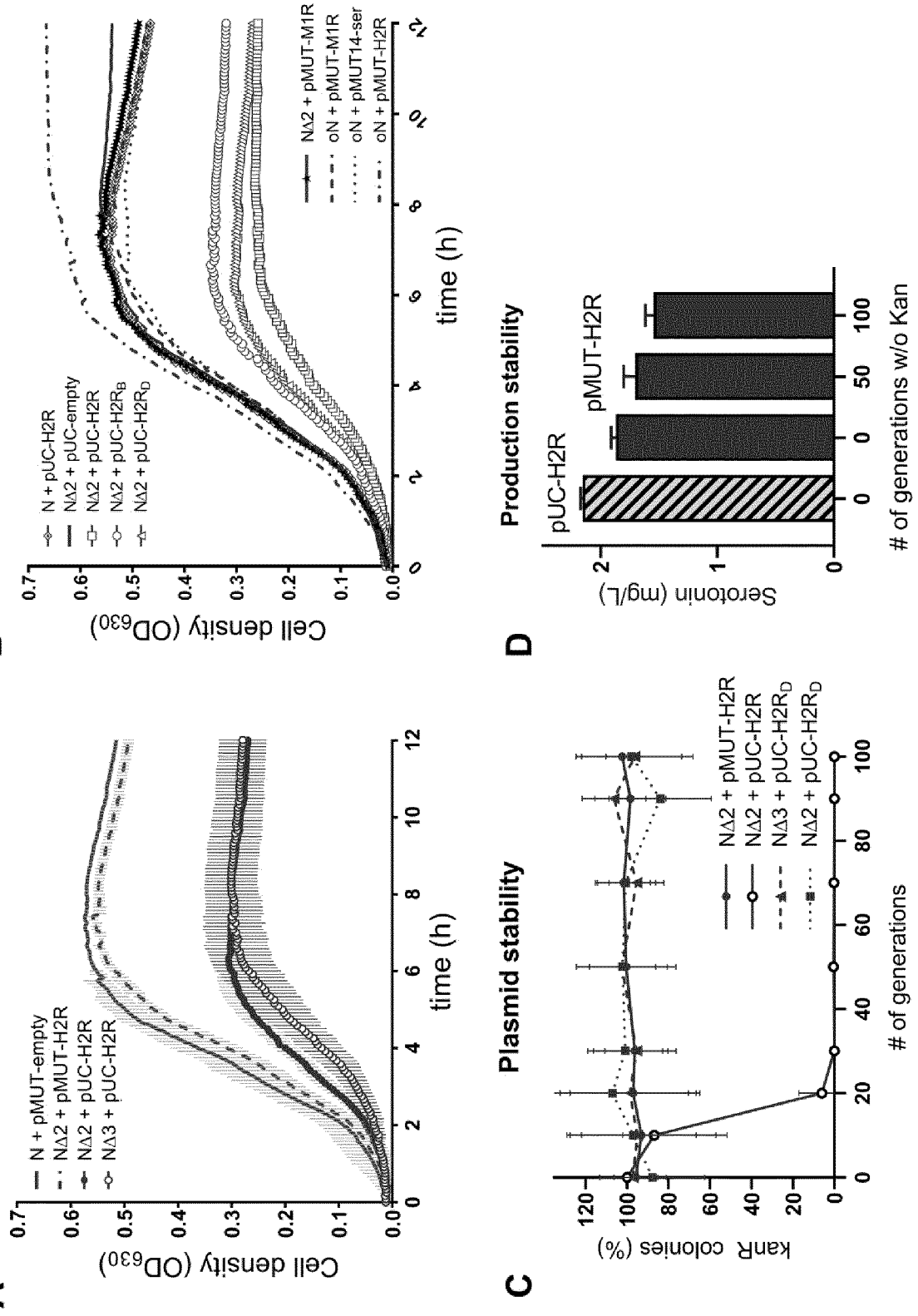

A
Cell density (OD630)
time (h)
N + pMUT-empty
NΔ2 + pMUT-H2R
NΔ2 + pUC-H2R
NΔ3 + pUC-H2R
B
Cell density (OD630)
time (h)
N + pUC-H2R
NΔ2 + pUC-empty
NΔ2 + pUC-H2R
NΔ2 + pUC-H2RB
NΔ2 + pUC-H2RD
NΔ2 + pMUT-M1R
oN + pMUT-M1R
oN + pMUT14-ser
oN + pMUT-H2R
C
Plasmid stability
kanR colonies (%)
# of generations
NΔ2 + pMUT-H2R
NΔ2 + pUC-H2R
NΔ3 + pUC-H2RD
NΔ2 + pUC-H2RD
D
Production stability
Serotonin (mg/L)
pUC-H2R
pMUT-H2R
# of generations w/o Kan

Figure 4

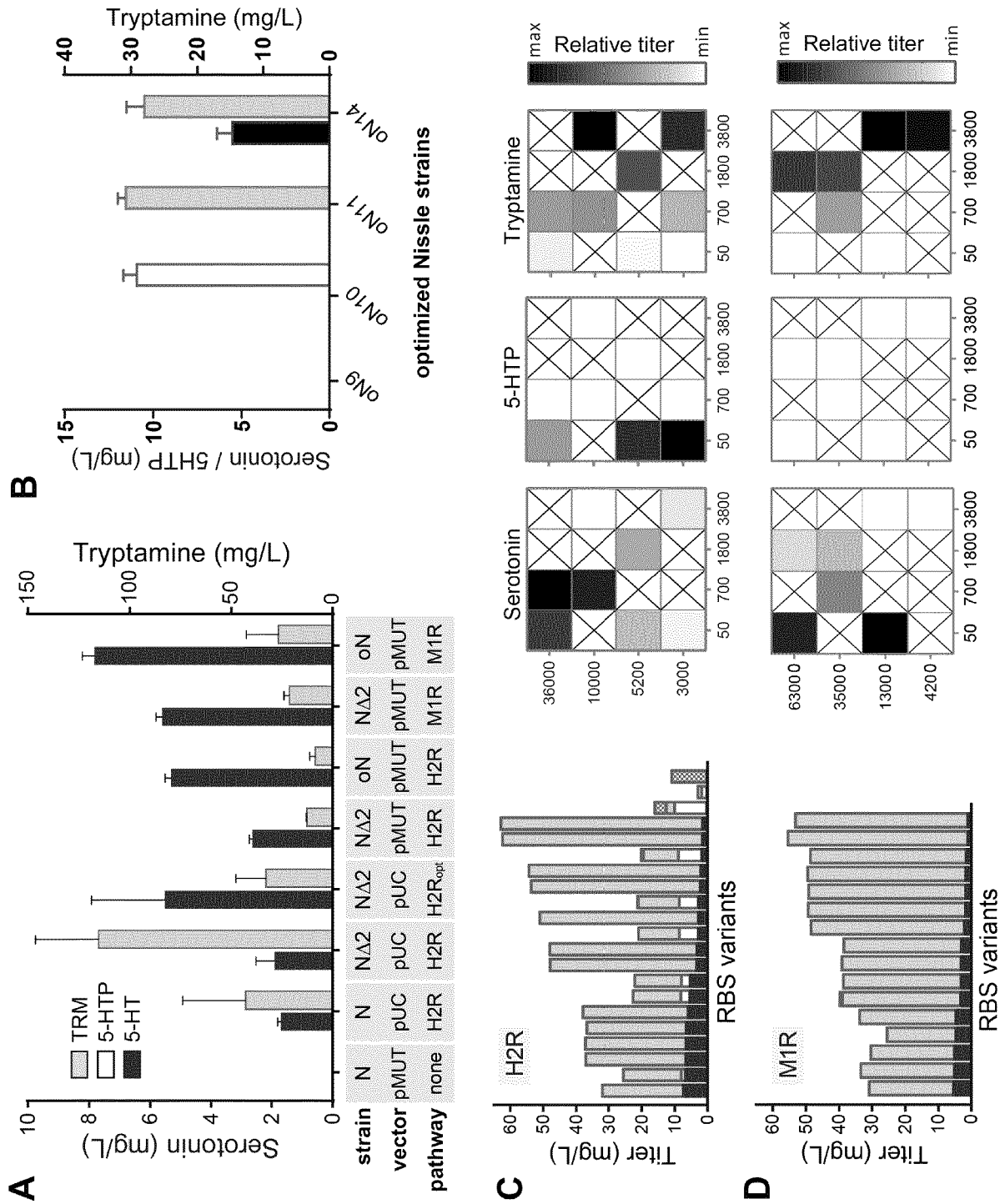

A
Serotonin (mg/L)
Tryptamine (mg/L)
TRM
5-HTP
5-HT
strain
vector
pathway
N pMUT none
N pUC H2R
NΔ2 pUC H2R
NΔ2 pUC H2R$_{opt}$
NΔ2 pMUT H2R
oN pMUT H2R
NΔ2 pMUT M1R
oN pMUT M1R
B
Serotonin / 5HTP (mg/L)
Tryptamine (mg/L)
ON9
ON10
ON11
ON14
optimized Nissle strains
C
H2R
Titer (mg/L)
RBS variants
Serotonin
5-HTP
Tryptamine
max
Relative titer
min
36000
10000
5200
3000
50 700 1800 3800
D
M1R
Titer (mg/L)
RBS variants
63000
35000
13000
4200

Figure 5

Figure 5 E

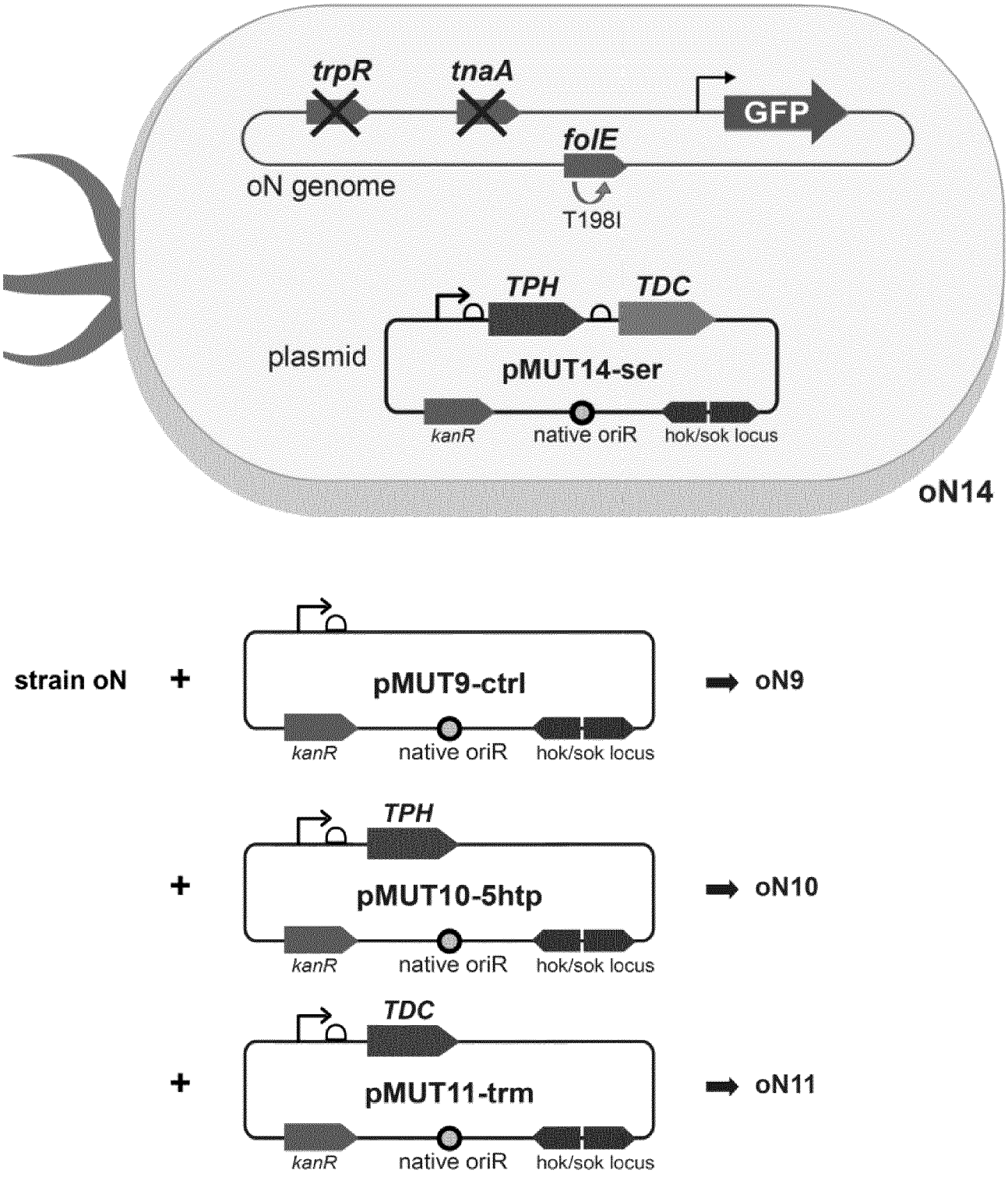

trpR
tnaA
GFP
folE
oN genome
T198I
TPH
TDC
plasmid
pMUT14-ser
kanR
native oriR
hok/sok locus
oN14
strain oN
+
pMUT9-ctrl
kanR
native oriR
hok/sok locus
oN9
TPH
+
pMUT10-5htp
kanR
native oriR
hok/sok locus
oN10
TDC
+
pMUT11-trm
kanR
native oriR
hok/sok locus
oN11

Figure 5F

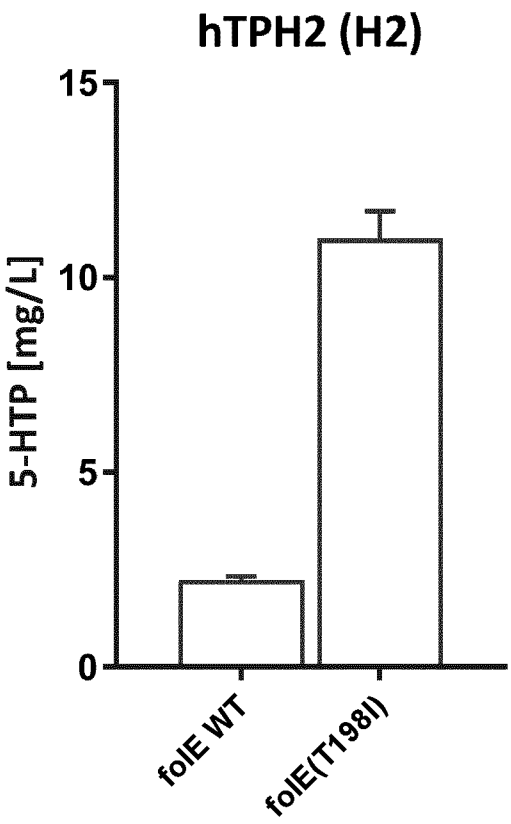


Figure 6 A

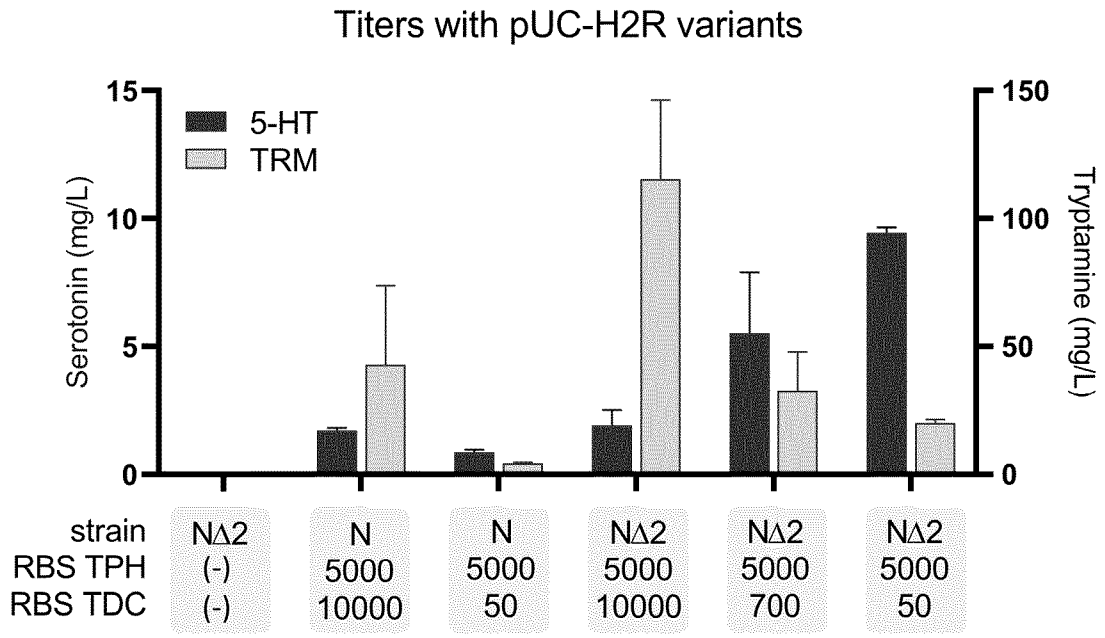


Figure 6B

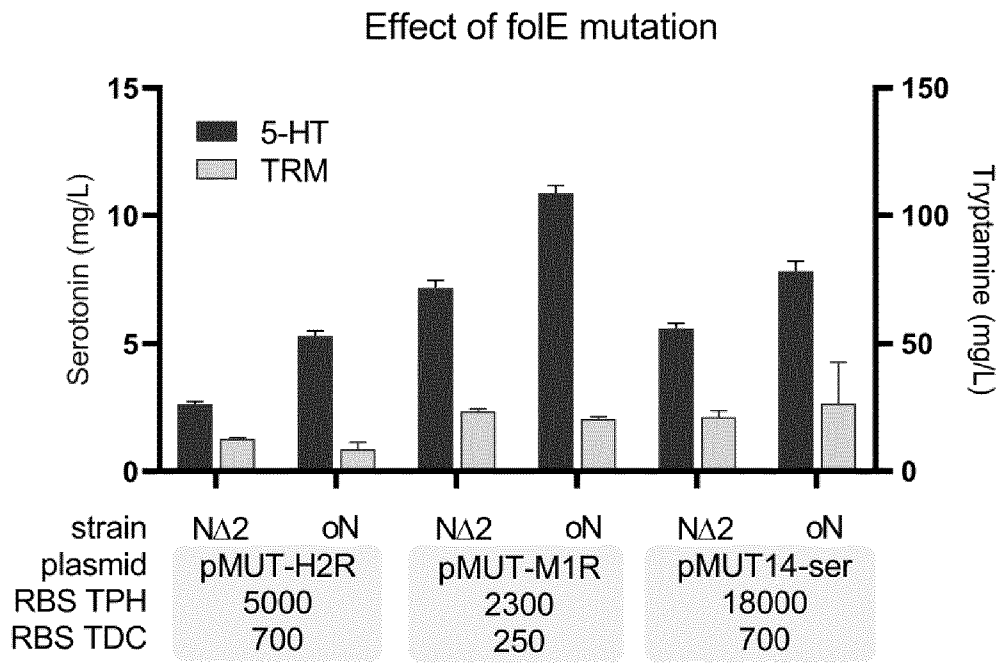
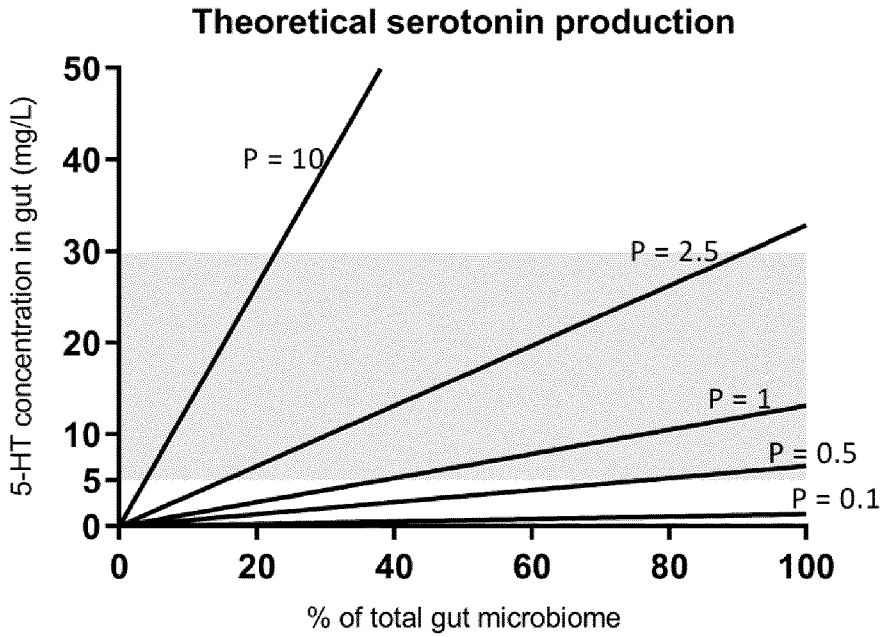
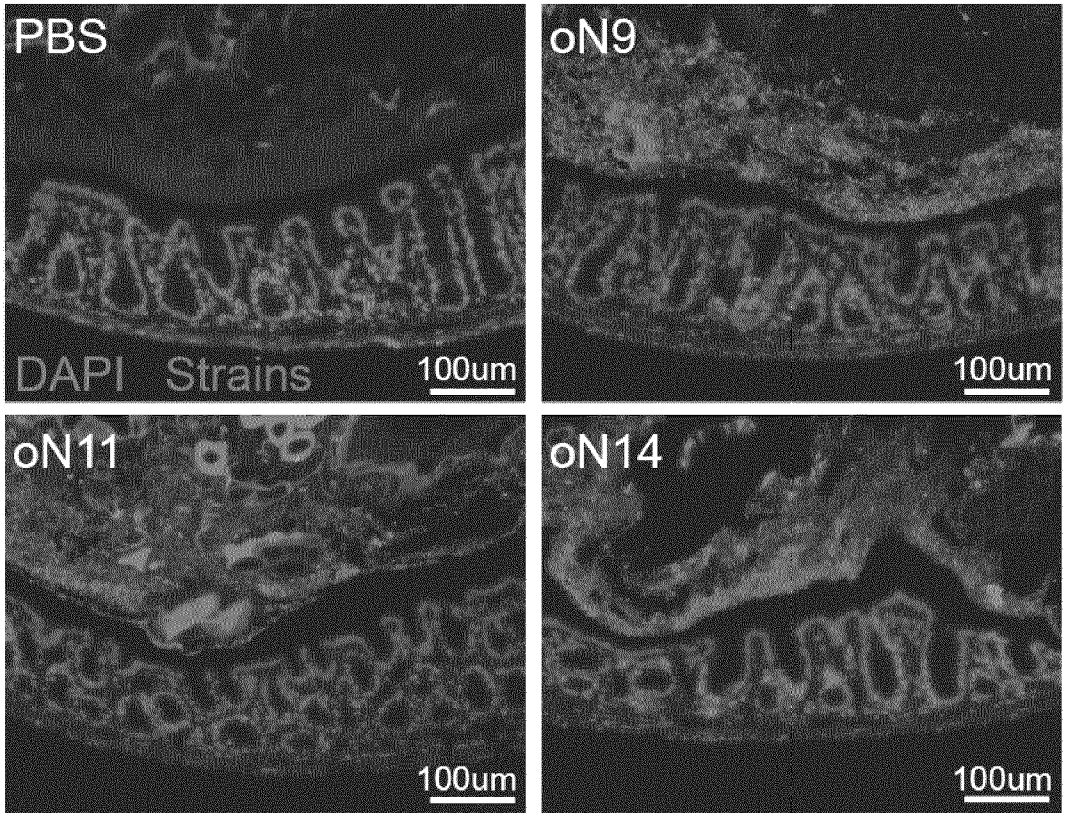


Figure 6C



Figre 7A



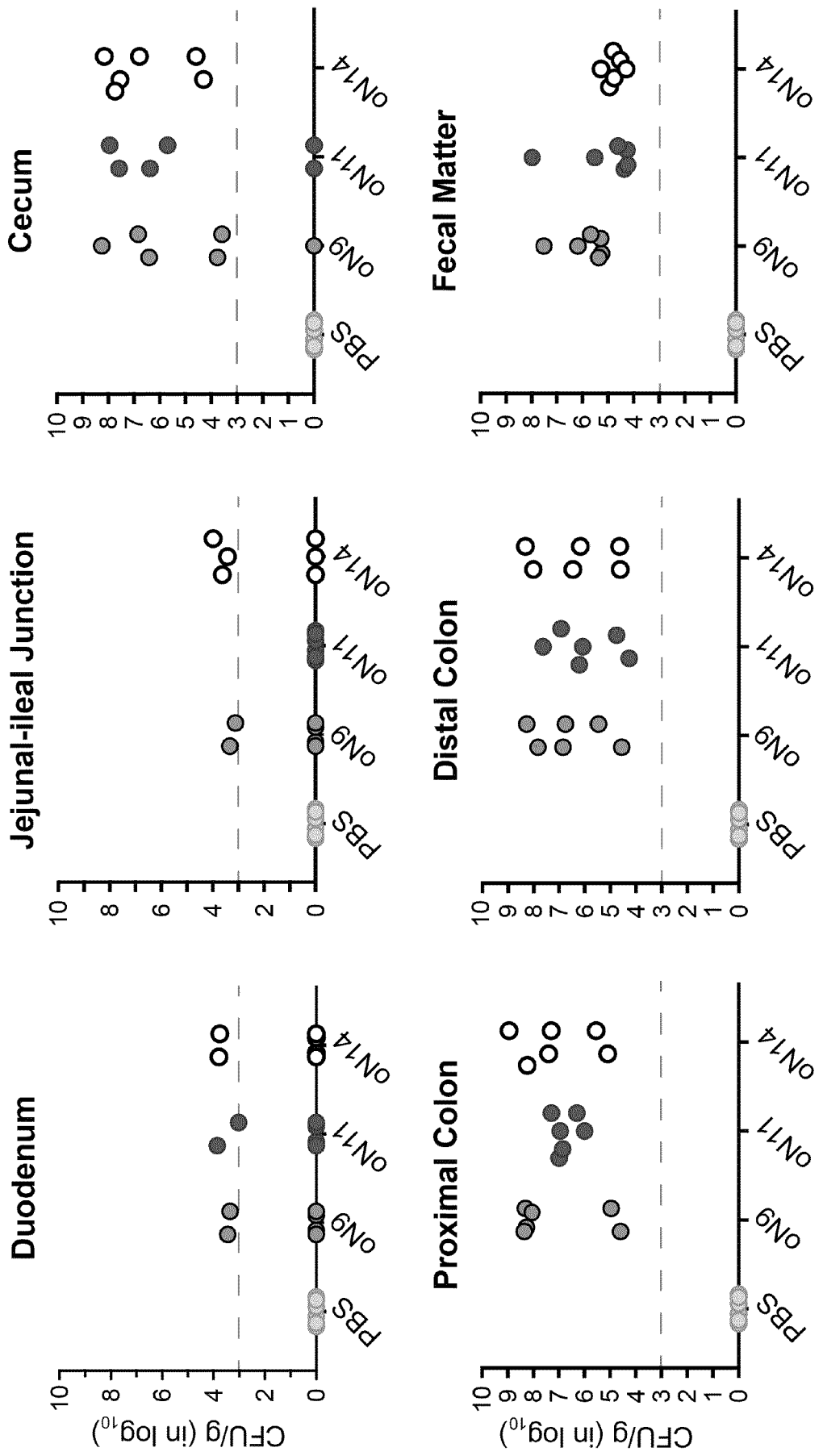
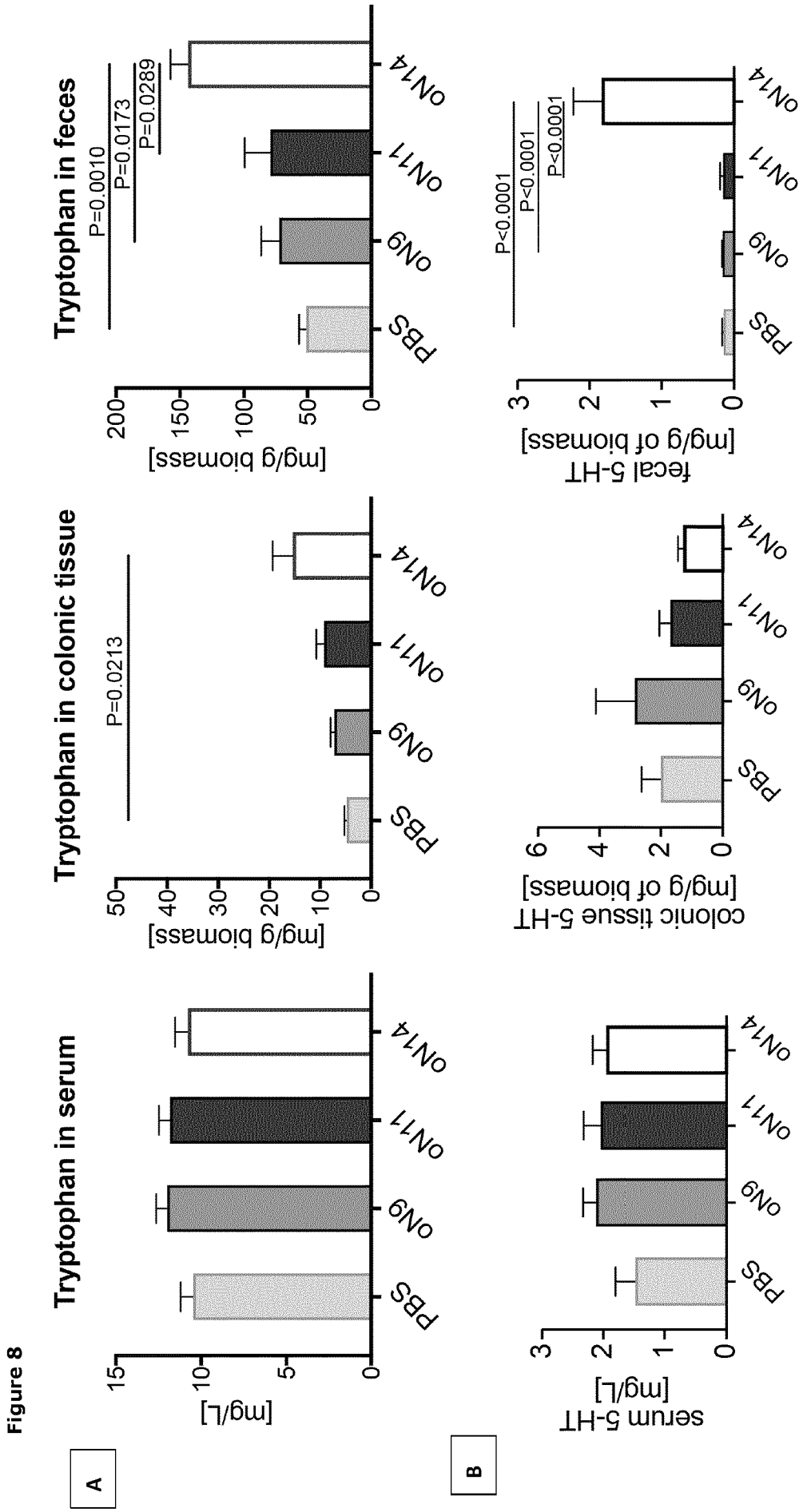
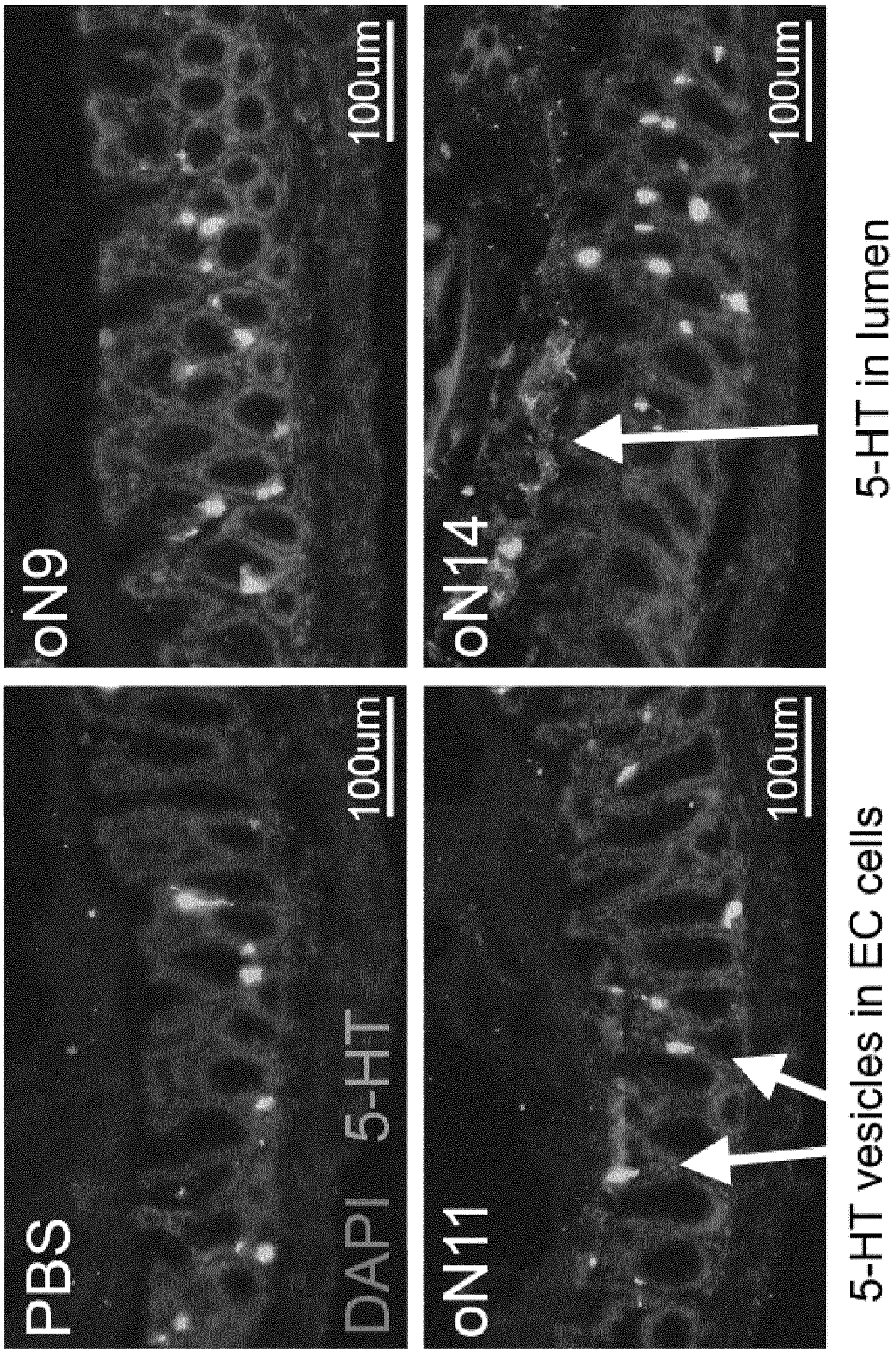

Duodenum
Jejunal-ileal Junction
Cecum
Proximal Colon
Distal Colon
Fecal Matter
CFU/g (in log10)
PBS
ON9
ON11
ON14

Figure 7B

Figure 8


A
Tryptophan in serum
[mg/L]
PBS
ON9
ON11
ON14
Tryptophan in colonic tissue
P=0.0213
[mg/g biomass]
Tryptophan in feces
P=0.0010
P=0.0173
P=0.0289
[mg/g biomass]
B
serum 5-HT
[mg/L]
colonic tissue 5-HT
[mg/g of biomass]
P<0.0001
P<0.0001
P<0.0001
fecal 5-HT
[mg/g of biomass]


PBS
DAPI 5-HT
100um
oN9
100um
oN11
100um
oN14
100um
5-HT vesicles in EC cells
5-HT in lumen

11

Figure 8C

Figure 9A

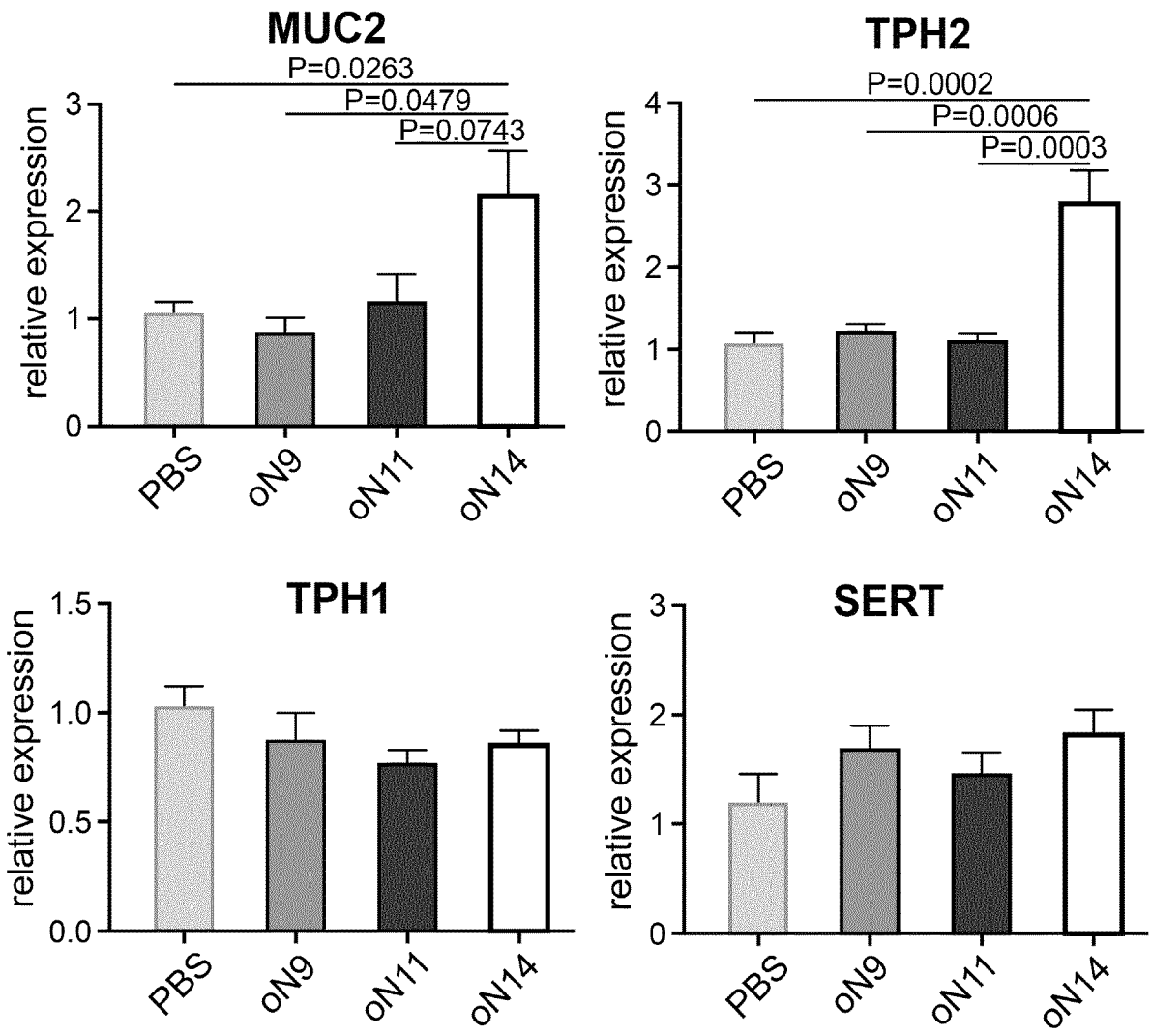

MUC2
P=0.0263
P=0.0479
P=0.0743
relative expression
0
1
2
3
PBS
oN9
oN11
oN14
TPH2
P=0.0002
P=0.0006
P=0.0003
relative expression
0
1
2
3
4
PBS
oN9
oN11
oN14
TPH1
relative expression
0.0
0.5
1.0
1.5
PBS
oN9
oN11
oN14
SERT
relative expression
0
1
2
3
PBS
oN9
oN11
oN14

Figure 9B

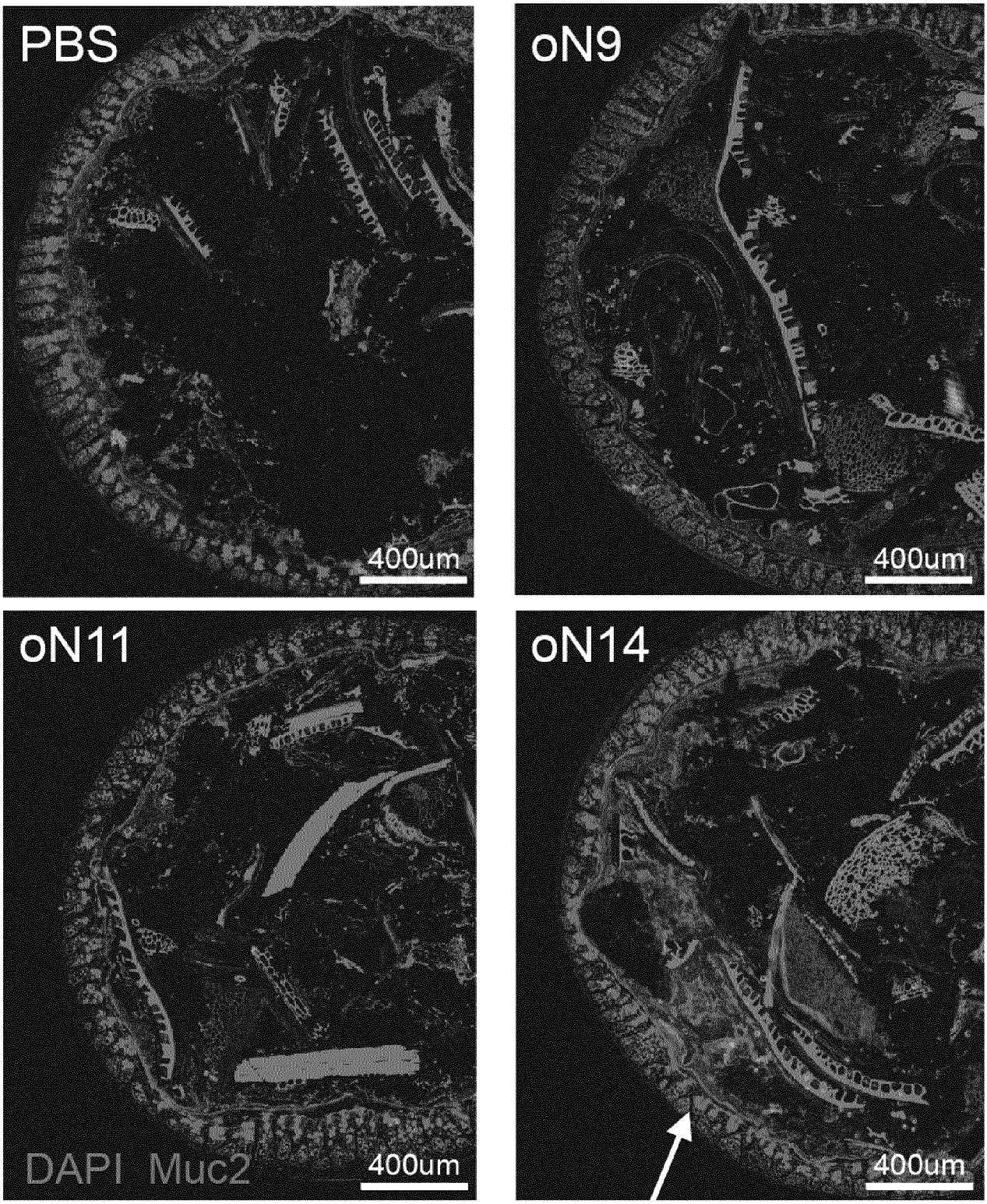
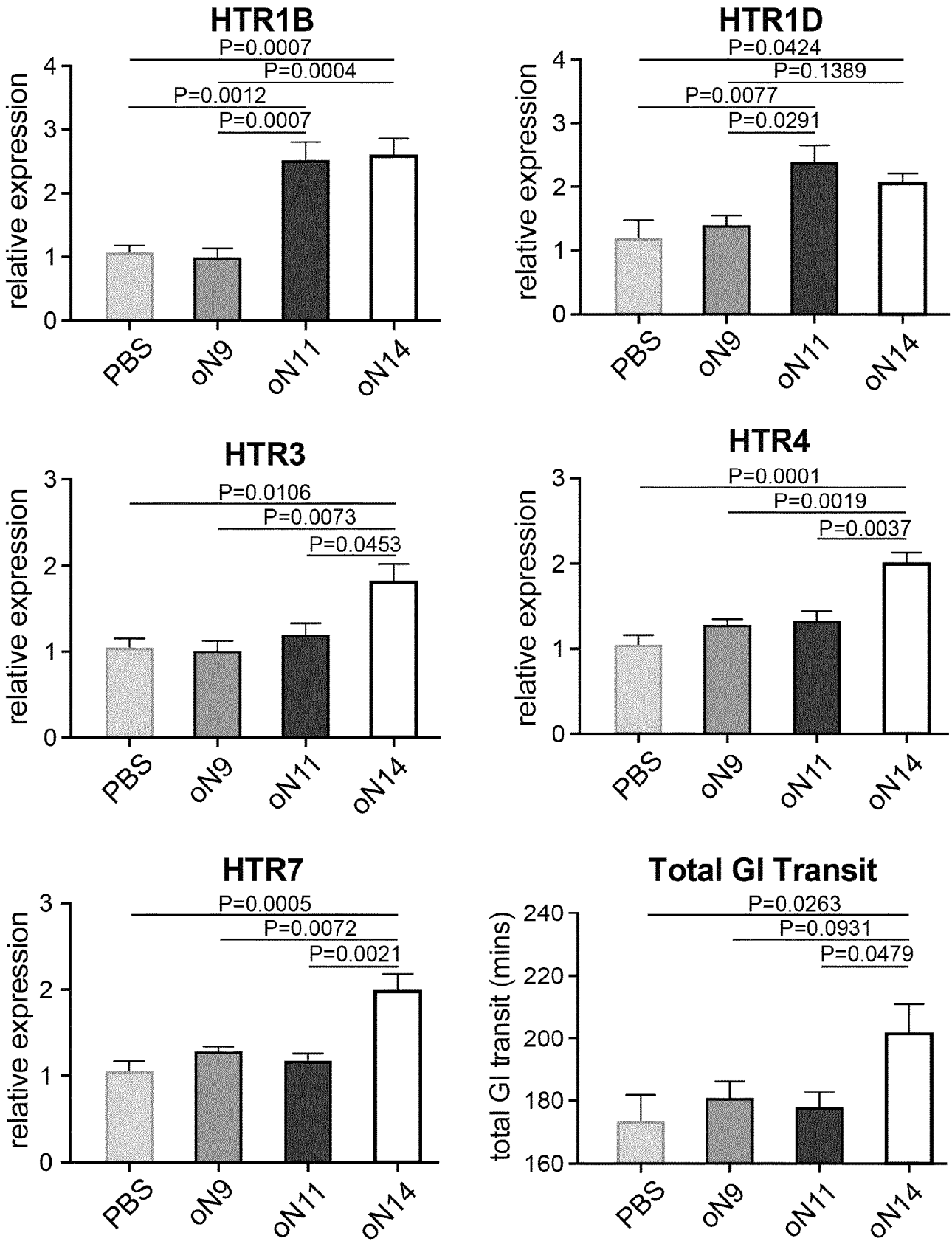


Figure 10


HTR1B
P=0.0007
P=0.0004
P=0.0012
P=0.0007
relative expression
0
1
2
3
4
PBS
oN9
oN11
oN14
HTR1D
P=0.0424
P=0.1389
P=0.0077
P=0.0291
relative expression
0
1
2
3
4
PBS
oN9
oN11
oN14
HTR3
P=0.0106
P=0.0073
P=0.0453
relative expression
0
1
2
3
PBS
oN9
oN11
oN14
HTR4
P=0.0001
P=0.0019
P=0.0037
relative expression
0
1
2
3
PBS
oN9
oN11
oN14
HTR7
P=0.0005
P=0.0072
P=0.0021
relative expression
0
1
2
3
PBS
oN9
oN11
oN14
Total GI Transit
P=0.0263
P=0.0931
P=0.0479
total GI transit (mins)
160
180
200
220
240
PBS
oN9
oN11
oN14

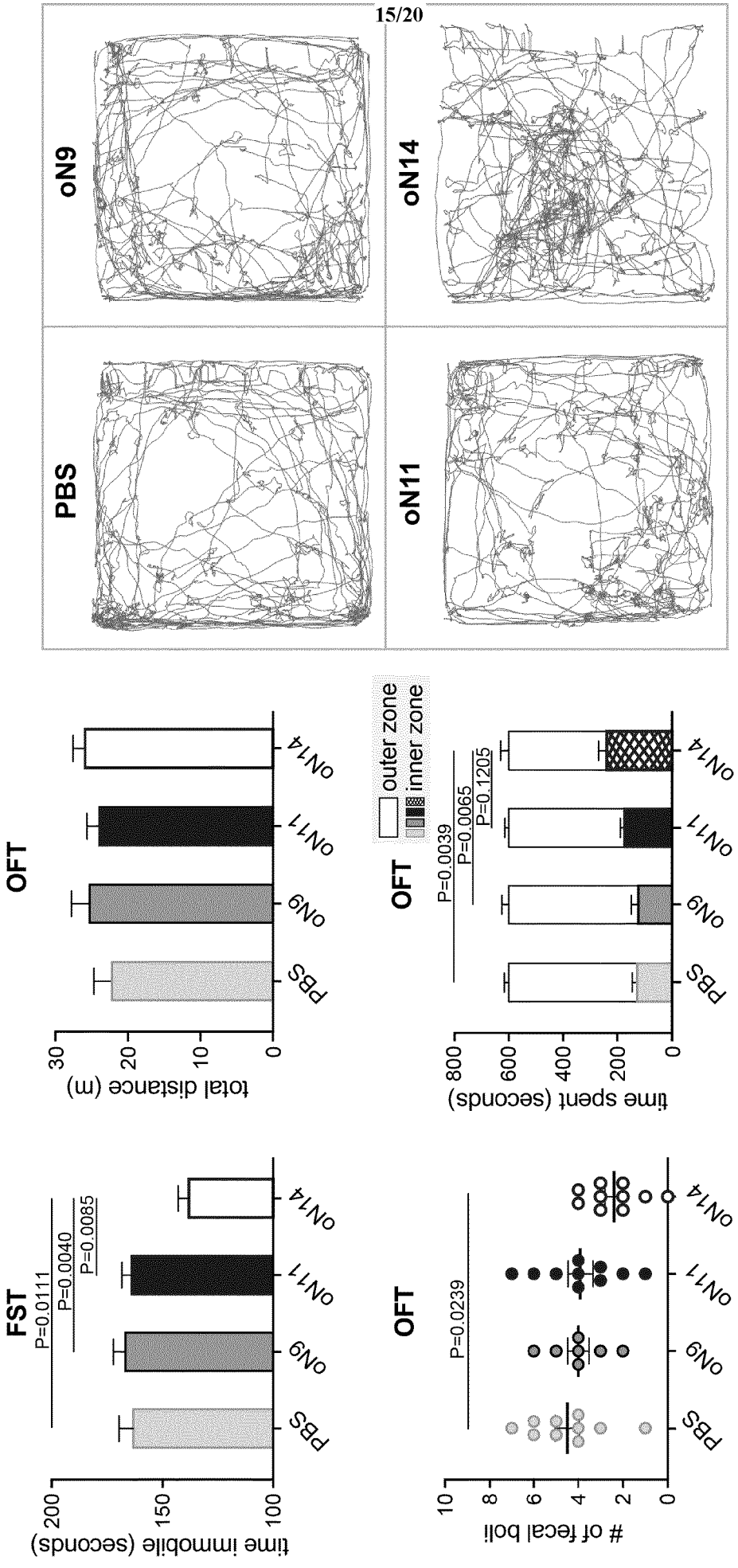

PBS
oN9
oN11
oN14
FST
P=0.0111
P=0.0040
P=0.0085
time immobile (seconds)
200
150
100
PBS
ON9
ON11
ON14
OFT
P=0.0239
# of fecal boli
10
8
6
4
2
0
OFT
outer zone
inner zone
P=0.0039
P=0.0065
P=0.1205
time spent (seconds)
800
600
400
200
0
OFT
total distance (m)
30
20
10
0

Figure 11

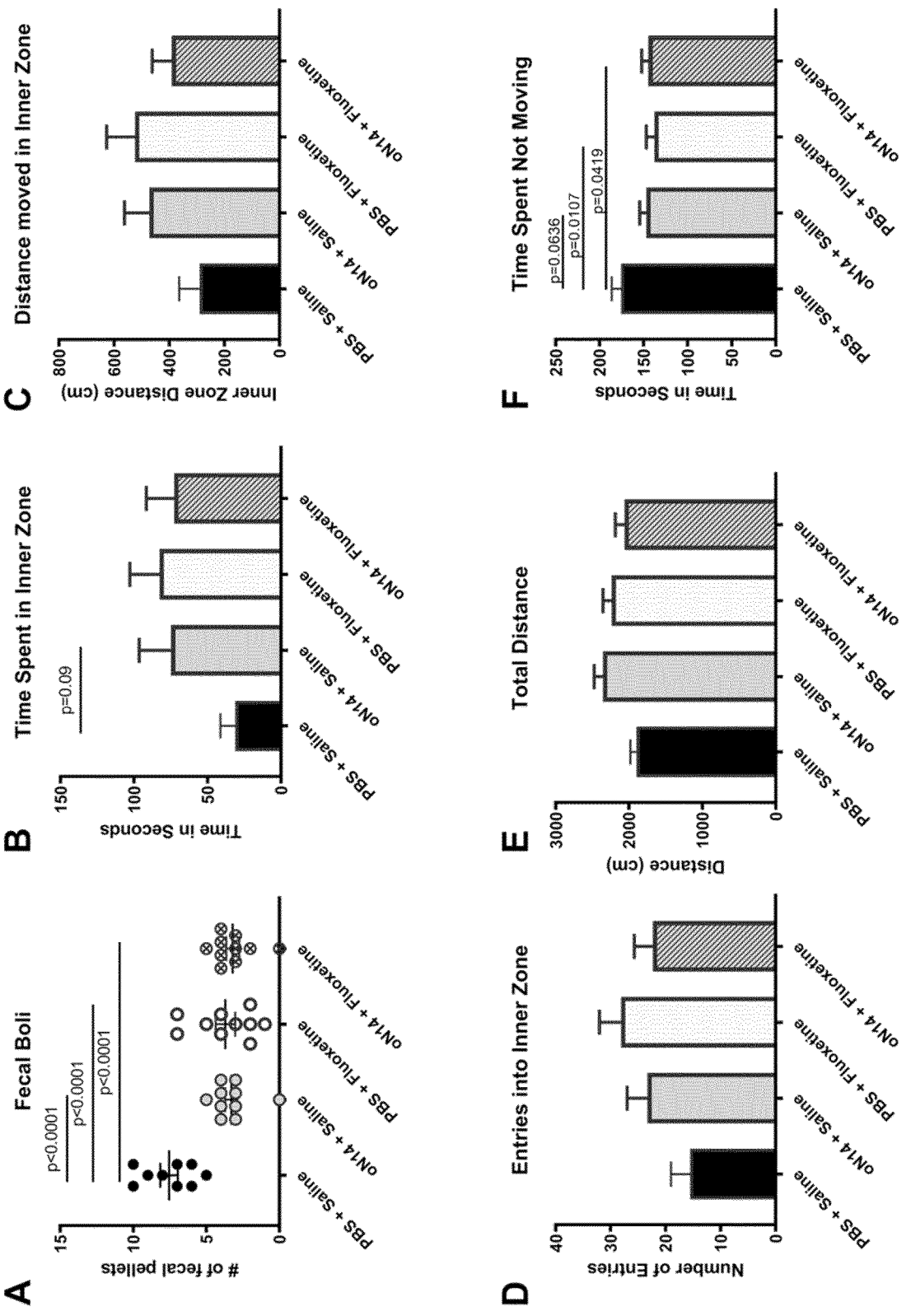

A
Fecal Boli
# of fecal pellets
p<0.0001
p<0.0001
p<0.0001
B
Time Spent in Inner Zone
Time in Seconds
p=0.09
C
Distance moved in Inner Zone
Inner Zone Distance (cm)
D
Entries into Inner Zone
Number of Entries
E
Total Distance
Distance (cm)
F
Time Spent Not Moving
Time in Seconds
p=0.0636
p=0.0107
p=0.0419
PBS + Saline
ON14 + Saline
PBS + Fluoxetine
ON14 + Fluoxetine

Figure 12

Figure 13

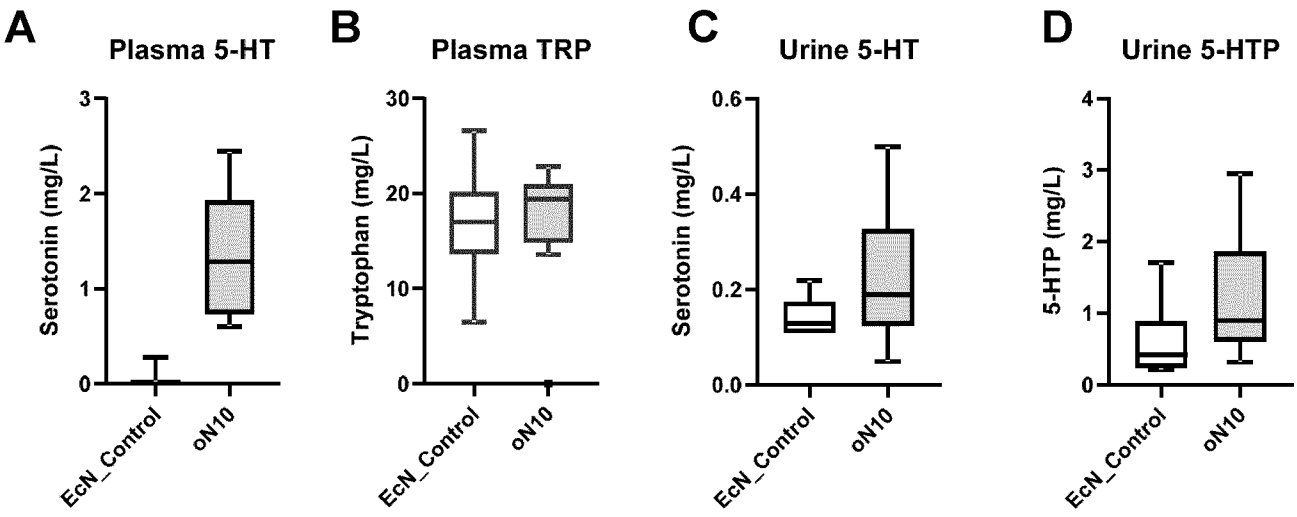

A
Plasma 5-HT
Serotonin (mg/L)
3
2
1
0
EcN_Control
oN10
B
Plasma TRP
Tryptophan (mg/L)
30
20
10
0
EcN_Control
oN10
C
Urine 5-HT
Serotonin (mg/L)
0.6
0.4
0.2
0.0
EcN_Control
oN10
D
Urine 5-HTP
5-HTP (mg/L)
4
3
2
1
0
EcN_Control
oN10

Figure 14

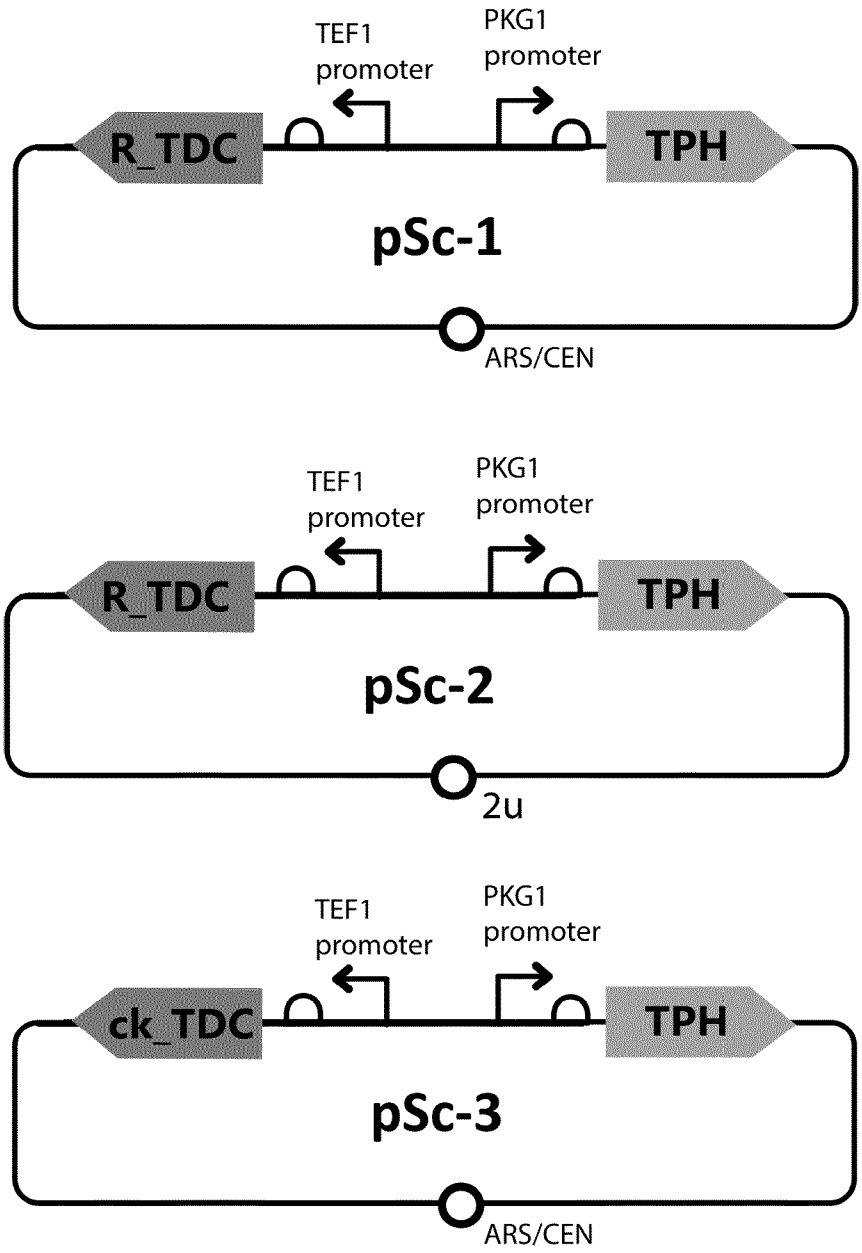

TEF1 promoter
PKG1 promoter
R_TDC
TPH
pSc-1
ARS/CEN
TEF1 promoter
PKG1 promoter
R_TDC
TPH
pSc-2
2u
TEF1 promoter
PKG1 promoter
ck_TDC
TPH
pSc-3
ARS/CEN

Figure 15

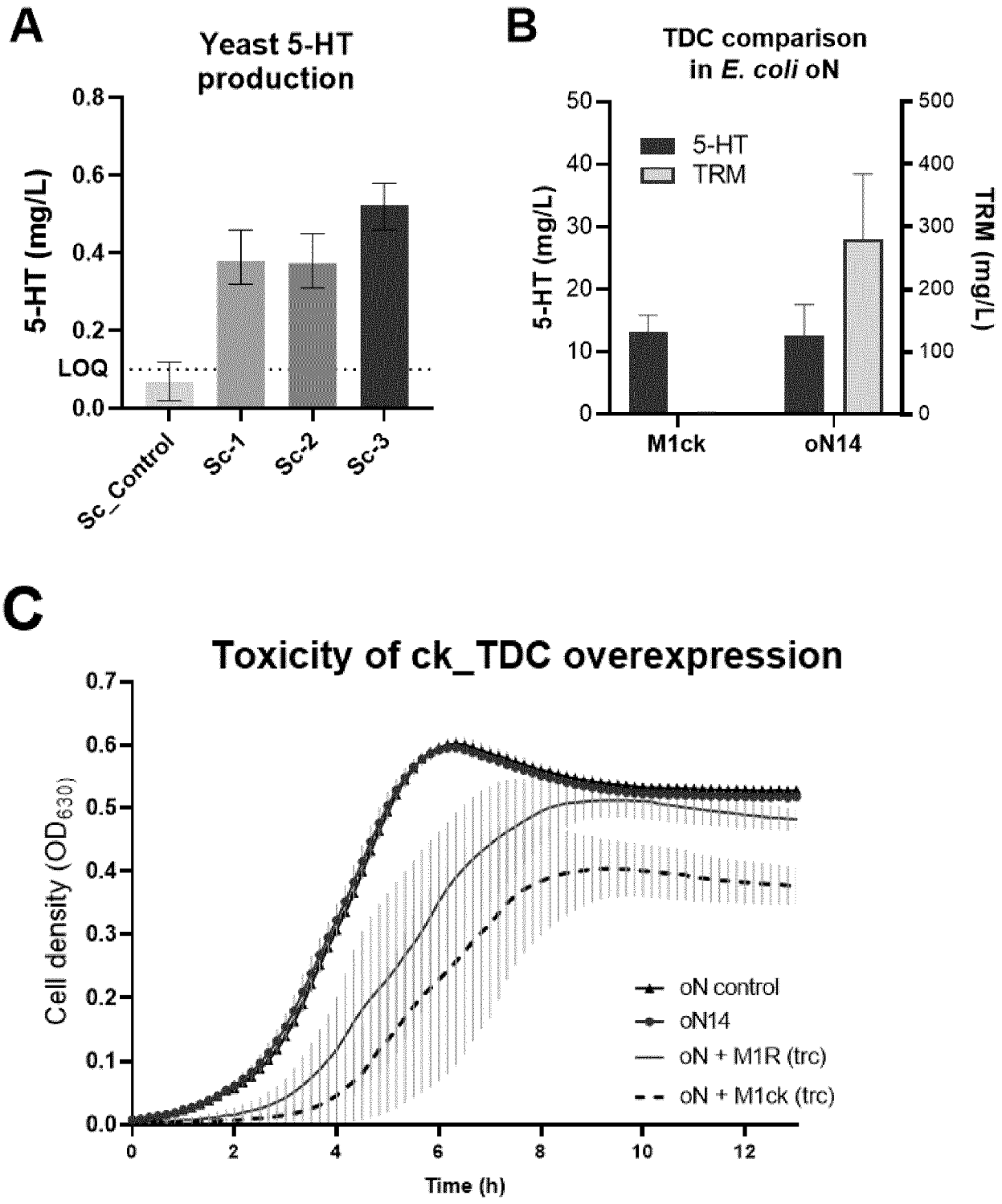
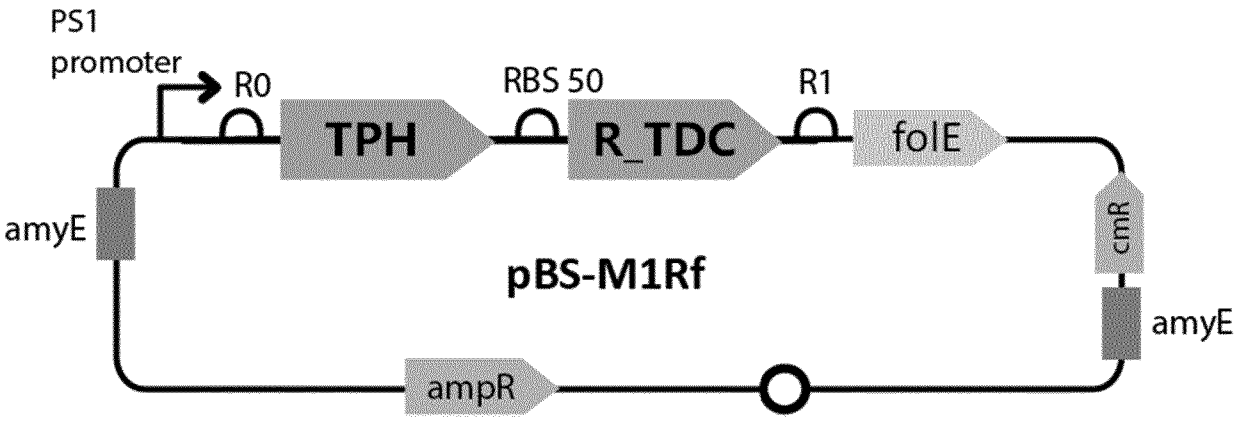

A
Yeast 5-HT production
5-HT (mg/L)
0.8
0.6
0.4
0.2
LOQ
0.0
Sc_Control
Sc-1
Sc-2
Sc-3
B
TDC comparison in E. coli oN
5-HT
TRM
5-HT (mg/L)
TRM (mg/L)
50
40
30
20
10
0
500
400
300
200
100
0
M1ck
oN14
C
Toxicity of ck_TDC overexpression
Cell density (OD630)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
Time (h)
oN control
oN14
oN + M1R (trc)
oN + M1ck (trc)

Figure 16


PS1
promoter
R0
TPH
RBS 50
R_TDC
R1
folE
amyE
pBS-M1Rf
cmR
amyE
ampR

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/066383

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 A61K35/74 C12N9/10 C12N9/78 C12N9/88
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, IBM-TDB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/136795 A1 (SYNLOGIC INC [US]) 10 August 2017 (2017-08-10)	1,2, 5-15, 19-21, 25-30
Y	the whole document in particular pages 1-3, [02]-[06]; page 7 lines 15-16; page 51 [100]-[104]; page 117, [0254]; page 149 [0311];page 191 [436]; page 192,[0442];page 416, [0893]; page 418, [0897]; page 531, [01141]	3,4, 16-18, 22-24
Y	WO 2013/127915 A1 (UNIV DANMARKS TEKNISKE [DK]) 6 September 2013 (2013-09-06) the whole document in particular Figs 1-3; page 8 line 21-page 9 line 18; page 78, [0158]; example 5; claims 1-19	3,16,22

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 August 2020	15/09/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dumont, Elisabeth

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2017/167866 A1 (UNIV DANMARKS TEKNISKE [DK]) 5 October 2017 (2017-10-05) the whole document in particular page 4 line 29-page 5 line 30; claims 1-20	11-13 4,17,18, 23,24
A	ZHI-JUN ZHAO ET AL: "Development of-tryptophan production strains by defined genetic modification in Escherichia coli", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY ; OFFICIAL JOURNAL OF THE SOCIETY FOR INDUSTRIAL MICROBIOLOGY, SPRINGER, BERLIN, DE, vol. 38, no. 12, 4 May 2011 (2011-05-04), pages 1921-1929, XP019982205, ISSN: 1476-5535, DOI: 10.1007/S10295-011-0978-8 the whole document in particular abstract; page 1926, col. 1 par. 1	1-15
A	HOWARD M SALIS ET AL: "Automated design of synthetic ribosome binding sites to control protein expression", NATURE BIOTECHNOLOGY, vol. 27, no. 10, 1 October 2009 (2009-10-01), pages 946-950, XP055062298, ISSN: 1087-0156, DOI: 10.1038/nbt.1568 abstract	1-15
A	Pedro Belda Ferre: "Engineering bacteria to treat genetic diseases", Medical Press, 27 November 2018 (2018-11-27), pages 1-3, XP055668339, Retrieved from the Internet: URL:https://medicalxpress.com/news/2018-11-bacteria-genetic-diseases.html [retrieved on 2020-02-13] the whole document	1-15

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/066383

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017136795 A1	10-08-2017	NONE	
WO 2013127915 A1	06-09-2013	EP 2820121 A1	07-01-2015
		US 2015024440 A1	22-01-2015
		WO 2013127915 A1	06-09-2013
WO 2017167866 A1	05-10-2017	EP 3436595 A1	06-02-2019
		WO 2017167866 A1	05-10-2017