

Methods for drug delivery

This application claims priority from 10202400040Q filed 05 January 2024, and from 10202400039Q filed 05 January 2024, the contents and elements of which are herein incorporated by reference for all purposes.

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Sequence Listing

The instant application contains a Sequence Listing which has been submitted herewith and is hereby incorporated by reference in its entirety. Said .xml copy, created on January 02, 2025, is named

10 008686008, and is 11,797 bytes in size.

Field of the Invention

The present invention relates generally to methods for drug delivery and more specifically to methods for drug delivery to the CNS using Lactobacillus bacteria engineered to produce and/or display the drug.

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Background

The blood-brain barrier (BBB) impedes the delivery of many pharmaceutical compounds to the brain (Alagangi et al., 2022). Intranasal administration through the olfactory epithelium (OE) presents an alternative direct pathway for brain-targeted therapeutic delivery, bypassing the BBB (Hanson and Frey, 2008; Trevino et al., 2020). In the nasal cavity, the OE serves as an interface between the central nervous system (CNS) and the external environment. Intranasally administered therapeutics can be transported across the OE to the olfactory bulb (OB) and other parts in the brain due to the unique anatomical properties of the OE. This avoids first-pass metabolism in the liver, thus increasing bioavailability in the brain and reducing systemic side effects.

25 Despite its benefits, this nose-to-brain delivery route can be limited by the anatomy and absorptive limitations of the OE. This is due to several reasons. Firstly, the OE constitutes only a small fraction of the surface area of the human nasal cavity—approximately 5 cm² or 3% of the total surface area (Gizurarson, 2012; Harkema et al., 2006). This significantly limits the amount of medication that can be absorbed and delivered to the brain and CNS compared to other routes of administration, such as oral and intravenous. 30 Secondly, the maximum volume for drug administration through the OE is about 200 µl (Pandey et al., 2020). While the nasal cavity has a total volume of about 6 cm³, most of intranasally administered medication ends up contacting and being absorbed by the respiratory epithelium (RE) and transported to the circulatory system instead. Mucociliary clearance and enzymatic degradation in the RE reduces the absorptive efficacy through the RE compared to the OE (Ganger and Schindowski, 2018; Keller et al., 35 2022). Lastly, the location of the OE at the roof of the nasal cavity requires the patient to be in an inverted position during and after drug administration, which can be inconvenient for the patient (Mittal et al., 2014).

There is thus a need for improved methods of nose-to-brain drug delivery that more effectively targets the OE and reduces dosing requirements for a range of drug modalities. It would be desirable to overcome or alleviate at least one of the above-described problems, or at least to provide a useful alternative.

The present invention has been devised in light of the above considerations.

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Summary of the Invention

The inventors made the surprising discovery that commensal microbes could be used for the intranasal delivery of therapeutics to the brain, specifically targeting the olfactory epithelium (OE) and the olfactory pathway. Intranasal delivery offers a pathway for administering medication directly to the brain, bypassing the blood-brain-barrier (BBB), but is impeded by the spatial constraints and limited capacity of the OE. Described herein is an engineered commensal Lp strain as a targeted vehicle for intranasal delivery, designed to increase precision and efficacy.

In a first aspect, the present invention provides an engineered *Lactobacillus* bacteria either (a) comprising an expression construct encoding a drug that is releasable from the bacteria and/or (b) carrying a drug that is releasable from the bacteria, wherein said drug can act upon the central nervous system (CNS) after passing the blood-brain-barrier (BBB). This includes, for example, drugs that can interact with targets in the brain and/or spine but are inhibited from accessing those targets by the BBB. Exemplary classes of agents include monoclonal antibodies, gene therapies, biologics, polar molecules, etc. In some embodiments, the present invention provides a method for bypassing the BBB. In some embodiments, the releasable drug may be secreted from the bacteria, for example by virtue of being fused to a secretion signal peptide that is also encoded by the expression construct. The drug may be expressed on a surface of the bacteria for example on a receptor or surface protein. The drug may be loaded onto the surface of bacteria, for example in the form of a prodrug such that it is carried by the bacteria.

In a second aspect, the present invention provides a method of delivering a drug to the central nervous system (CNS) of a subject, the method comprising intranasally administering a *Lactobacillus* bacteria engineered to produce and/or carry the drug to the subject, wherein the drug is releasable from the bacteria for delivery to the CNS. The drug may be secreted from the bacteria or expressed on the surface of the bacteria.

In some embodiments of the engineered *Lactobacillus* bacteria of the first aspect or the method of the second aspect, the *Lactobacillus* bacteria is *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus casei*, *Lactobacillus delbrueckii* or *Lactobacillus rhamnosus*. In preferred embodiments, the engineered *Lactobacillus* bacteria is *Lactobacillus plantarum* (Lp). In further preferred embodiments, the Lp is of the strain WCFS1. In some embodiments, the bacteria are engineered to secrete the drug, for example by a secretion signal peptide. In some embodiments, the bacteria are administered intranasally to the nasal cavity. In some embodiments, the bacteria are administered by nasal spray, nasal drops, nasal rinse, inhalation device, exhalation delivery system or nasal implant. In some embodiments, the bacteria comprise an OppA polypeptide on a surface of the bacteria. This OppA polypeptide may be an

exogenous polypeptide that is not expressed by non-engineered bacteria or an endogenous polypeptide that is expressed by non-engineered bacteria. The OppA polypeptide may be a purified or semi-purified preparation that is added to the bacteria and coated on the surface of the bacteria. The OppA polypeptide may be covalently or non-covalently attached to a surface of the bacteria. The OppA polypeptide may be
5 attached to the cell wall or cell membrane of the bacteria. The bacteria may contain one or more OppA polypeptides. In further embodiments, the OppA polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 1–7. These OppA polypeptides may bind *Lactobacillus* and heparan sulphate (NaHS), including for example, the apical NaHS expressed on neuronal cilia.

10 In some embodiments, the drug is a small molecule, peptide, polypeptide or a combination thereof. In some embodiments, the drug is a chemical messenger. This includes for example, pheromones, neurotransmitters, hormones or neuro-peptides. In some embodiments, the drug is a peptide. In preferred embodiments, the peptide is a hormone. In some embodiments, the hormone is any one of Leptin, α -MSH, BDNF or any combination thereof. In preferred embodiments, the hormone is Leptin. In some
15 embodiments, the drug is a prodrug.

In some embodiments, the bacteria localise to the olfactory epithelium (OE) of the subject. In further embodiments, the bacteria bind to apical NaHS on neuronal cilia in the subject. In further embodiments, the released drug traverses the OE and reaches the olfactory bulb (OB) in the subject. The drug traverses by the process of diffusion. In further embodiments, the drug traverses the lamina propria (LP) in the
20 subject. In further embodiments, the drug traverses olfactory nerve bundle (ONB) in the subject. In further embodiments, the drug traverses the cribriform plate (CP) in the subject. In yet further embodiments, the drug traverses through the perineural space in the subject. In preferred embodiments, the drug diffuses from the OB to the rest of the brain. In some embodiments, the drug is administered in (a) a daily-dosing regimen, or (b) an alternate-day dosing regimen. In some embodiments, the subject is a
25 mammal. For example, a mouse, monkey or human. In preferred embodiments, the subject is a human suffering from a disease.

In a third aspect, the present invention provides a method of treating a disease or disorder of the central nervous system (CNS) in a subject, the method comprising intranasally administering an effective amount of a *Lactobacillus* bacteria engineered to produce and/or carry a drug to the subject. In some
30 embodiments, the drug is releasable from the bacteria for delivery of the drug to the CNS to treat the disease or disorder. In some embodiments, the disease or disorder is a chronic disease or disorder. In some embodiments, this is a disease or disorder of the brain. In other embodiments, the disease or disorder is a metabolic disease or disorder. In further embodiments, the metabolic disease or disorder is obesity. In some embodiments, the disease or disorder may be a neurodegenerative disease or disorder.
35 In preferred embodiments, this includes Parkinson's disease or Alzheimer's disease.

In a fourth aspect, described herein is a *Lactobacillus* bacteria engineered to produce and/or carry a drug, for use in treating a disease or disorder of the central nervous system (CNS) in a subject, wherein an

effective amount of the bacteria is to be administered intranasally to the subject. In some embodiments, the *Lactobacillus* bacteria is the *Lactobacillus* bacteria of the first aspect.

In a fifth aspect, described herein is a *Lactobacillus* bacteria engineered to produce and/or carry a drug in the manufacture of a medicament for treating a disease or disorder of the central nervous system (CNS)
5 in a subject, wherein an effective amount of the bacteria is to be administered intranasally to the subject. In some embodiments, the *Lactobacillus* bacteria of the first aspect.

In a sixth aspect, described herein is a pharmaceutical composition for intranasal administration, comprising a *Lactobacillus* bacteria engineered to produce and/or carry a drug. In some embodiments, the composition comprises *Lactobacillus* bacteria of the first aspect. In some embodiments, the
10 pharmaceutical composition of comprises one or more *Lactobacillus* bacteria. In some embodiments, the pharmaceutical composition comprises a first bacteria, a second bacteria and a third bacteria according to the first aspect, in which the first bacteria is engineered to produce and/or carry a payload comprising Leptin and the second bacteria is engineered to produce and/or carry a payload comprising α -MSH and the third bacteria is engineered to produce and/or carry a payload comprising α -MSH.

In a seventh aspect, described herein is a kit comprising the bacteria of the first aspect. In some
15 embodiments, the kit comprises a first container containing a first composition according to the sixth aspect, and a second container containing a second composition according to the sixth aspect, in which the drug expressed and/or carried by the bacteria of the first composition comprises a different drug to the drug expressed and/or carried by the bacteria of the second composition. For example, the first bacteria
20 may express and/or carry leptin, and the second bacteria may express and/or carry α -MSH. In further embodiments, the kit may also comprise a third or more containers containing a third or more compositions according to the sixth aspect, in which the drug expressed and/or carried by the bacteria is BDNF.

In an eight aspect, described herein is a method of delivering a drug to the brain of a subject, wherein the
25 method comprises the bacteria of the first aspect.

The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

Summary of the Figures

30 Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures in which:

Figure 1 shows intranasal delivery of recombinant appetite-regulating hormones by engineered Lp. a) Lp specifically localises to the olfactory epithelium through the recognition of NaHS. b) Lp displays surface oligopeptide substrate binding (OppA) proteins that exhibit binding affinity to NaHS. c) Engineered Lp
35 produces and secretes three appetite-regulating hormones: Leptin, α -MSH (α -Melanocyte-stimulating

hormone), and BDNF (Brain-derived neurotrophic factor). d) Hormones secreted by Lp are translocated from the OE to the OB within the brain.

Figure 2 shows flow cytometric analysis of binding capacity of nasal commensal strains to NS0 cells. a) Schematic diagram illustrating the binding of FITC-labeled bacteria to NS0 cells. b) Binding capacity of commensal strains to NS0 cells.

Figure 3 shows the identification of NaHS binding protein from Lp. a) Molecular structure of NaHS. b) Schematic diagram showing the immunoprecipitation (IP) assay used to identify NaHS binding protein from Lp. c) Identification of the NaHS binding protein, Lp_0783, through IP. d) & e) Flow cytometric assessment of the binding affinity of OppA proteins to CHO-K1 cells and NS0 cells.

Figure 4 shows targeted localisation of Lp in mouse olfactory epithelium. a) Schematic diagram showing the intranasal administration of FITC-labelled Lp. b) Schematic diagram showing the location of various mucosa in the mouse nasal cavity. OB = olfactory bulb, OE = olfactory epithelium, SE = squamous epithelium, RE = respiratory epithelium, NP = nasopharynx, CTX = cortex. c) & d) IVIS images showing the localization of Lp in the OE from a dorsal or lateral angle. The color scale represents the range of radiant efficiency (outlined by white squares). e) & f) Quantitative analysis of total radiant efficiency of FITC in the RE and OE.

Figure 5 shows targeted delivery of FITC to the OE by Lp and FITC accumulation in the lamina propria. a) IF staining showing a targeted deliver of FITC to OE by Lp. Blue – Hoechst, Green – FITC, Red – anti CNGA2. White arrows indicate the areas magnified in Figure 3c. Scale bar: 300 µm. b) Magnified image IF images showing the gradual diffusion and subsequent concentration of FITC in the lamina propria and nasal septum (NS). Scale bar: 100 µm.

Figure 6 shows the enrichment of FITC in the around olfactory nerve bundles in the lamina propria under the OE. a) Schematic diagram showing the relative position of the coronal sections. b) Schematic diagram delineating various pathways through which biomolecules translocate from the nose to the brain: i) biomolecules travel through the OE. ii) biomolecules travel through the lamina propria. 1 – Paracellular pathway (between epithelial cells); 2 – Transcellular pathway (through epithelial cells); 3 – Intracellular pathway (through olfactory neurons). c). IF staining of nasal cavity coronal sections in depth showing a targeted delivery of FITC to OE by Lp after 14-days of administration. Scale bar: 300 µm. White rectangles indicate the magnified regions. White arrows indicate the FITC labeled Lp. d) Magnified image IF images showing the gradual diffusion and subsequent accumulation of FITC in the lamina propria and nasal septum near the OE. Scale bar: 100 µm. e) Magnified IF images showing the enrichment of FITC around the olfactory nerve bundles in the lamina propria under the OE. White triangles indicate the olfactory nerve bundles. Scale bar: 25 µm. Fluorescence staining: Blue — Hoechst, Green — FITC, Red — anti- OMP. Abbreviations: CSF = cerebrospinal fluid, LaP = lamina propria, NS = nasal septum, OEC = olfactory ensheathing cells, OB = olfactory bulb, OE = olfactory epithelium, ONB = olfactory nerve bundle, ONF = olfactory nerve fibroblasts, RE = respiratory epithelium, VNO = vomeronasal organ.

Figure 7 shows translocation of FITC from the lamina propria to the OB. a) IF staining showing the accumulation of FITC in the lamina propria under OE, following 3-, 7- and 14-days administration. Blue — Hoechst, Green — FITC, Red - anti-OMP. Scale bar: 100 μ m. b) IF staining showing the accumulation of FITC near the CP over time and the diffusion of FITC to the OB across the CP (top panel). Blue —871 Hoechst, Green — FITC, Red — anti-OMP. White arrows indicate the accumulation of FITC near the CP and its entry to the OB through the perineural spaces. Scale bar: 100 μ m. IF staining showing the accumulation of FITC in the OE, the lamina propria and the CP over time and the diffusion of FITC to the OB across the cribriform plate (CP) (bottom panel). Blue – Hoechst, Green – FITC, Red – anti-CNGA2. White arrows indicate the accumulation of FITC near the CP and its entry to the OB through the perineural spaces. Scale bar: 100 μ m. c) IVIS images of the mice brains (dorsal) showing a gradual accumulation of FITC in the OB. The colour scale indicates the range of radiant efficiency, $3.25e7 - 4.20e7$ (p/s) / (μ W/cm²). d) Quantification of the total radiant efficiency of FITC in the OB. Error bars indicate the standard error. Unpaired two-sided Student's t-tests were performed to determine statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. e) Schematic diagram showing the OB (*) and hypothalamus (Δ). f) IVIS images of mouse brains (lateral) showing the dispersal of FITC from the OB to other parts of the brain. The color scale indicates the range of radiant efficiency, $1.80e7 - 3.50e7$ (p/s) / (μ W/cm²).

Figure 8 shows secretion and bioactivity of appetite-regulating hormones in Lp. a) Schematic diagram showing the mechanism of action of the hormones. b) Western blot analysis verifying the secretion of recombinant leptin, and its bioactivity, indicated by the phosphorylation of Stat. c) Western blot analysis verifying the secretion of recombinant BDNF, and its bioactivity, indicated by the phosphorylation of TrkB. d) ELISA assay detecting the secretion of α -MSH and verifying its bioactivity by measuring the levels of intracellular cAMP. Error bars indicate the standard error. Unpaired two-sided Student's t-tests were performed to determine statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 9 shows translocation of recombinant appetite-regulating hormones by Lp. a) Concentrations of FITC, Leptin, α -MSH, and BDNF in the transwell and basal compartment after administration of engineered Lp strains during a 72-hour co-culture. b) Transport efficiency of FITC, Leptin, α -MSH, and BDNF during a 24-hour co-culture. (top panel). c) IF images displaying the transport of Leptin, α -MSH, and BDNF to the OE at 24- and 48-hours post-administration of the corresponding Lp strains. Scale bars: 100 μ m (top panel). d) IF images at higher magnification, highlighting the supporting cells in the OE 24 hours post administration. Scale bars: 25 μ m (lower panel). Blue – Hoechst, Green – anti-Leptin, α -MSH, or BDNF. Error bars represent the standard error. Statistical significance was assessed using unpaired two-sided Student's t-tests. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 10 shows intranasal delivery of appetite-regulating hormones by engineered Lp in obese mouse models. a) Schematic diagram showing the intranasal administration of engineered Lp. b) Comparative analysis of body weight gain among mice across different groups over an eight-week period. c) Average daily food intake by the mice. d) Fasting glucose levels measured in the mice after seven weeks of treatment. e) Glucose response test in the mice after seven weeks of treatment. f), g) & h) Endpoint

subcutaneous, peri-gonadal, and peri-renal fat ratio in mice. i) Endpoint liver TAG level in mice. j) Oil Red O staining of liver large lobes from the mice. Scale bar: 20 μ m. Lp-Mix = mixed regimen of all three engineered strains. Error bars indicate the standard error. Unpaired two-sided Student's t-tests were performed to determine statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

- 5 **Figure 11** shows a comparison of intranasal delivered Leptin secreting Lp and recombinant leptin in lean mice under high fat diet. Figure 8. Comparison of intranasal delivered Leptin secreting Lp and recombinant leptin in lean mice under high fat diet. a) Schematic diagram showing the dosage of various treatment groups. b) IF images showing the absence of Leptin in the OE at 24- and 48-hours post-administration of recombinant leptin. Scale bars: 100 μ m (top panel). Scale bars: 25 μ m (lower panel). c)
- 10 Comparative analysis of body weight gain among mice across different groups over an eight-week period. d) Average daily food intake by the mice. $n = 3$ cages. e) Fasting glucose levels measured in the mice after seven weeks of treatment. f) Glucose response test in the mice after seven weeks of treatment. g), h) & i) Endpoint subcutaneous, perigonadal, and peri-renal fat mas in mice. Abbreviations: Lp-EV = empty vector; r-Lep and r-Lep-LD refer to daily and alternative day administration of recombinant Leptin; Lp-Lep and Lp-Lep-LD refer to daily and alternative day administration of engineered *L. plantarum* strains
- 15 secreting Leptin, representatively. $n = 6$ mice unless otherwise stated. Individual data points are indicated in color circles. Error bars indicate the standard error. Statistical significance was assessed using unpaired two-sided Student's t-tests. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

20 **Detailed Description of the Invention**

Aspects and embodiments of the present invention will now be discussed with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

- This invention harnesses commensal probiotic bacteria to achieve high target specificity for nose-to-brain drug delivery. The inventors identified commensal *Lactobacillus* strains with natural affinity for the OE and showed that the bacteria can be engineered to function as a vector for drug delivery to the CNS. For example, the *Lactobacillus* bacteria can produce and secrete recombinant appetite-regulating hormones to alleviate obesity-related symptoms when administered intranasally in a murine model of obesity. Appetite-regulating hormones act on the hypothalamus and play a crucial role in maintaining energy
- 30 balance.

- As a crucial interface for external exposure, the nasal cavity harbours a diverse range of microorganisms, with the densest microbiome in the respiratory system. Without being bound by theory, it is postulated that commensal bacteria in the nasal cavity have the potential to serve as a delivery vector for precision therapy targeting the OE and brain. Previous studies have revealed the presence of a gut microbiome-brain axis that forms a bidirectional connection between the gastrointestinal tract and the CNS. Compared
- 35 to the gut microbiome, microbes in the nasal cavity, especially in the olfactory mucosa, are anatomically closer to the brain and potentially have more intimate crosstalk with the CNS through the exchange of

metabolites. With targeted localisation on the OE, *Lactobacillus* strains of this disclosure can direct therapeutics precisely to the OE and olfactory pathway and provide an enduring treatment effect through constant secretion of therapeutics.

5 The *Lactobacillus* bacteria of this disclosure may express OppA proteins, which exhibit a high affinity for heparin sulphate (NaHS), enabling specific localisation within the OE by binding to apical NaHS on neuronal cilia. This OE-specific localisation ensures that therapeutics released by Lp can be transported to the brain via the olfactory pathway, preventing unintended distribution to the respiratory epithelium and the circulatory system. Furthermore, extended localisation of the bacteria in the OE over several days following intranasal administration can provide continuous release of therapeutics in the OE to
10 maintaining therapeutically relevant levels of drugs in the CNS. This would reduce the frequency of dosing.

Accordingly, this disclosure provides methods for drug delivery to the central nervous system (CNS) through intranasal administration of commensal bacteria engineered to produce and/or carry the drug. The drug is releasable from the bacteria for delivery to the CNS. The commensal bacterium is preferably
15 one that is capable of homing to and persisting on the olfactory epithelium, most preferably a non-pathogenic lactic acid bacterium, e.g., a *Lactobacillus sp.* bacterium, a *Bifidobacterium sp.* bacterium, or a *Lactococcus sp.* bacterium. Also provided are engineered commensal bacteria and pharmaceutical compositions containing the bacteria for use in the methods herein.

Disclosed herein is a method of delivering a drug to the central nervous system (CNS) of a subject, the
20 method comprising intranasally administering a *Lactobacillus* bacterium engineered to produce and/or carry the drug to the subject, wherein the drug is releasable from the bacterium for delivery to the CNS.

Disclosed herein is a *Lactobacillus* bacterium that expresses intrinsic OppA proteins, which exhibit a high affinity for NaHS, enabling specific localization within the OE by binding to apical NaHS on neuronal cilia.

25 Engineered bacteria

Provided herein are lactic acid bacteria engineered to produce and/or carry a drug for intranasal drug delivery to the olfactory region, brain and CNS. In preferred embodiments, the lactic acid bacteria are *Lactobacillus* strains.

The *Lactobacillus* bacterium may be a component of the native microbiome of nasal mucosa or tissue. In
30 some embodiments, the *Lactobacillus* bacterium is *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus casei*, *Lactobacillus delbrueckii* or *Lactobacillus rhamnosus*. In one embodiment, the *Lactobacillus* bacterium is *Lactobacillus plantarum* (Lp). In one embodiment, the *Lactobacillus* bacterium is *Lactobacillus plantarum* WCFS1.

In one embodiment, the bacterium is engineered to secrete the drug, preferably at an intranasal site (e.g.,
35 the olfactory epithelium) following intranasal administration. The bacterium may comprise one or more expression constructs encoding a heterologous drug molecule (e.g., a recombinant peptide or polypeptide) or encoding components of a biosynthetic pathway required for synthesis of a heterologous

drug molecule. The expression construct may comprise one or more nucleotide sequences encoding a peptide or polypeptide, operably linked to one or more of the same or different promoters capable of directing expression of the sequences in a *Lactobacillus* host. A promoter employed in accordance with the present methods is preferably expressed constitutively in the bacterium. The use of a constitutive
5 promoter avoids the need to supply an inducer or other regulatory signal for expression to take place. The promoter may also be an inducible promoter, preferably a promoter that is inducible under conditions found at an intranasal site. The promoter may be homologous to the bacterium employed, i.e., one that is found natively in that bacterium. Preferably, the promoter directs expression at a level at which the bacterial host cell remains viable, i.e., retains some metabolic activity, even if growth is not maintained.

10 The expression construct may comprise a secretory signal sequence. Preferred secretory signal sequences include any of those with activity in Gram-positive organisms, most preferably in *Lactobacillus*. Preferably, the drug is constitutively secreted.

In one embodiment, the bacterium is engineered to carry the drug. The drug is preferably carried on a surface of the bacterium, and is releasable from the bacterial surface for delivery to the CNS. For
15 example, the bacterium may be engineered to display a capture domain on a surface of the bacterium that is capable of binding the drug. The capture domain may be an oligonucleotide, peptide, polypeptide, small molecule, or any combination thereof, preferably a polypeptide, e.g., a protein or protein fragment. The capture domain may be native to the *Lactobacillus* bacterium or it may be a heterologous entity. The capture domain may contain an anchor domain for anchoring to the bacterial cell membrane or cell wall.
20 The capture domain may be capable of binding to the drug directly or to a binding partner on the drug. In one embodiment, the capture domain is a biotin-binding polypeptide, and the binding partner on the drug is biotin. The biotin-binding polypeptide may be endogenous or non-endogenous to the *Lactobacillus* bacterium. The biotin-binding polypeptide may be multimeric streptavidin, e.g., tetrameric streptavidin.

In one embodiment, the bacterium is engineered to secrete and carry the drug. The bacterium may
25 express and secrete the drug, after which the drug is displayed on the cell surface, e.g., through an anchor domain on the drug that anchors to the bacterial cell membrane or cell wall.

In some embodiments, the bacterium comprises an OppA polypeptide on a surface of the bacterium.

In one embodiment, the OppA polypeptide is an endogenous polypeptide that is expressed from the genome of the *Lactobacillus* bacterium. In another embodiment, the OppA polypeptide is a heterologous
30 polypeptide that is expressed from a heterologous nucleic acid introduced into the bacterium. In yet another embodiment, the OppA polypeptide is an exogenous polypeptide that is not expressed by the bacterium. The OppA polypeptide may be a purified or semi-purified preparation that is added to the bacterium and coated on the surface of the bacterium. The OppA polypeptide may be covalently or non-covalently attached to a surface of the bacterium. The OppA polypeptide may be attached to the cell wall
35 or cell membrane of the bacterium. The bacterium may contain one or more OppA polypeptides.

In one embodiment, the OppA polypeptide comprises an amino acid sequence having at least 70% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 1–7. These OppA sequences

bind both the *Lactobacillus* bacterium and heparan sulphate. In some embodiments, the OppA polypeptide comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 1–7. The bacterium may contain one or more OppA polypeptides. In preferred embodiments, the bacterium contains two or more OppA polypeptides, such as two, three, four, five, six or seven OppA polypeptides containing an amino acid sequence having at least 70% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 1–7. These OppA sequences bind both the *Lactobacillus* bacterium and heparan sulphate. In preferred embodiments, the bacterium contains two or more OppA polypeptides, such as two, three, four, five, six or seven OppA polypeptides containing an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 1–7.

Table 1. Exemplary OppA proteins (signal peptide underlined; added Myc tag in bold)

OppA protein	Amino acid sequence
Lp_0018 [UniProtKB - F9US48 (F9US48_LACPL)]	<u>MKKRIWISSIAIVALGLVLTGCGSQSSQ</u> QKTLNVTATQSIATADPNK ADDIASQAAIAQFMEGLYTTNQKGKVPAIAKKVVKPTNNGKTYTFNL RHNAKWSNGKKVTAADFVYSVQRQVDPNTKSQEVGHVAEIKNATAI TAGKKAVKTLGVKALGKYKLQITLQQRVPYFNYRLATEIYPLNKQFTE KYGNKYGTSAQRTLNGPYVLKSWNGTNDTWKYVKNPYYDRKQV RIKNIKVQTVKNNSTAENLFSNEVQVTQITGTQVSAAEQGSLKSQM KVTCLNQLYFMLWNQKRSALQNTDLRRVSYALNRQSLVKNVLKDG SVAATSLVPAGNTKNPTTGKDFNTDTGNLYPYSPAKAKQYWQKAQA SLGKQKLTQLLTNDNDINKSVAEYIQAAIEKNLSGVTNVKSVPLTN EISTLSKGDFFATLSWSSDFQDPVDFLNKASITNSVNFGEFNNAKY EQLISTITADKQSTKARYQTMQQAACLVAQQQGVTPYQTAAAHLSN KVGGVHFTLLRDALYRYAYWKE QKLISEEDL (SEQ ID NO: 1)
Lp_0092 [UniProtKB - F9USS1 (F9USS1_LACPL)]	<u>MSLSGTHKLILTIGSVVIIGGIGFTVNA</u> HSHPATTKQSQVINLSAGSEIM SLDPAKMTDSVSNSQVTQVDEGLYRLNSQSQPVNALATKTTVTNGG HTYIIQLRH DARWSNGNRVTAHDFVYSWRRTLDPKSKSEFTYVFTNI KNADAIAAGKKKPSTLGVKANGDYQLTITLSKPVAYFKKILAVSTFPV NQPAVNKYGGKYGTSAAATTVYNGPFTLTGWTGTNNTWTLKKNPAYY DHKLVLKHQINYQVKSNTTAYNLYQSNKLDVTLNGEQSVQNKNQ ELKTLAGGQISFIQYNHHDHVAANRELRTALSLAINRSQTLNKVLENG SLPAKSFQVRNMAKNPKTGTDVFNDATVDGTVNYNLNQARAAYHKA QRQLGKKSLLTITCGDDDNSHQVAEFIQGQLTSHLPGLKVTIKAMPF TAMLGKVSQKAFQLNLTSSWMDFADPSQSLTILTGDSNSNMGHYQS KAYDQAMQAADGPDALNATKRYQDLVQAAKTALTDQAVTPLYEGRS

	HLLVKSHLKGVIYNNFSGAANYRTAYVKEQKLISEEDL (SEQ ID NO: 2)
Lp_0200 [UniProtKB - F9UT07 (F9UT07_LACPL)]	<u>MKWKVMLTTATAAA</u> STGLLLAACSQGQSKSDNAGNLRAGMAQKQV LNWSENGSELTTLDTSLVTDISISANMINNTMEGLYRIGNNNKITPGIAT HTVISKDKKTYTFTLRKDAKWSNGDPVTAQDFVYSWRRTIDPKTASQ YAYLYSGIKNADQITNGKAKANSLSGIKADGKYKLTVTLDKPMAYFKLL MGFAIFFPQNHAVQKYGKDYGTATKMVYDGPFTMKGWKGTNTT WSLNRNPKYWDKKHVYLEKINDQVVKSTTTGFNLFQSNKLDMATLS GEQVKNEKNPKLVLRKTSRLNYLEFNQKKVPALANAKLRQAMSLTI DRHQLVTDVLADGSAPVPGFVTTGLATDPTTGEDFATENAVSAATT QDTAKAKKLWAEGLKETGKKSLLTLTHDNIDQTKETAIEYVQGQLEK ELPGLKITDITLPFKNRLARETSGNFQLAISGWQADFADPISDLGILTS TNDYNFGKWKKNADYDAAVKQAEQSTDPTVRWTALGKAEKIIGTDEG VAPLTQTTLAQMVNPKLKGIIYNTSGTNYNFKDAYMAKEQKLISEED L (SEQ ID NO: 3)
Lp_0201 [UniProtKB - F9UT08 (F9UT08_LACPL)]	<u>MQWKLFATVATLTATTG</u> VLLVCGCGSSSSSSNAGNTTAKMAKTQVL NWSENGQDLTTLDPSLATDVISGTMISNSQEGLYRLGKNSKVTPGIA TKTTVSKDKKTYTFTLRKNAKWSNGDQVTAQDFVYGWRRTVNPKTA SQYAYLYSGIKNADKIVSGKAKTSTLGIKADGKYKLTVKLEKPMQYFK LLMGFVFFPQNHAVEKYGKDYGTSSSKMVYNGPFKMTGWKGTN STWTLKRPNYWDKKHVYLTIKDQVVKSTTTAFNLFQSNKLDMATL SGQQVKNEKNNKLVRKSSRLNYLEFNQKKVKLLANKKIRQAMSLT LNRKQLVNDVLGDGSVTPKGFVTTGLATDPTTGKDFATENTVKSAVT QDTTKAKKLWKQGLKETGKKSLLTLTHDDTDQMKALAEYVQGQLE KELPGLKVQSVTPYKTRISREIAGNFQLVISAQADFADPVSDLGIM TSTNDYNFGKWTNKTFDSTANTSTSTTKRWQALATAEKTMTAD QGVAPLSQNVIAQMVNPKLKGIVYNTAGINYNFKSAYMAKEQKLISE EDL (SEQ ID NO: 4)
Lp_0783 [UniProtKB - F9UM05 (F9UM05_LACPL)]	<u>MKKRSVLAVVMSLVLT</u> VLAACGKHNSQSSSGNGKYASSQVLNLSYP SSLDSIDISNMSGYGSTGNIFESLYRLGKNGSITPGLAKSTKVSKDGK TYTFTIRNAKWSDGSKITAQDFVYSWKRTVTPATKSQYAYLFSGVKN ADEIVAGKKSPSTLGVKAQGEHTFIVTLDKPITYFKKLMTYPLFGPISE KAVKKWGNKYATKAQYMLYSGPFKLTGWTGTNNSWQFVKNNQYW DKKAVHLQKINYTVNESTTTTLNLFQEKKDLTQLASEQVKNMKSSS DYTTYPSITAFVYNFQDSNATIKKALNNAKIRQAISLSINRKTLVKNV IGDASTVSKTFVPQDLVKDAKTGKDFADESTVKNSTSYNKALAQKLW KQGLKETGIKKLSIQLLASNDEPNKPISQYLKSALEKNLDGLTVNLSNI PSKVASSRAQSGDFDLYLSGWGADFNDPISHLQIMTNNSGYNYGKY

	NSSTYNALVNKAQNQDANDTSARWQDMINAEKIMKDQGITPLYQT VYSYLQNPVKVGGIIHNTAGTQWNYKYAYIAKE EQKLISEEDL (SEQ ID NO: 5)
Lp_1261 [UniProtKB - F9UN51 (F9UN51_LACPL)]	<u>MNFKTAAKVTVVAGASVLF</u> <u>LAACGSSKSSSSSKQTANWTESAELPT</u> MDISKSTDVVSSNALNNTNEGLYRLGKNGKVTPGIAKSTKVTNGGKT YTFTLRKNAKWSNGDKVTAKDFVYGWQRTVNPKTASQYAYLYSGIK NADAIVNGKKPVSSLGKADGDYKLTVTLDKPIAYFDKLMGFVNFPPQ NEKVVKKYGSKYGTSSKAMVYNGPYKMTGWTGTNLSTWTLKKNNDY WDKKAVKMDSIKYQVVKDPSTGLNLFNSNKLDFTTLSNEQVKNYKN DKNYLVKRKTASTFYVEFNKDSKTLAKAFQNKKIRQALSLAINRKS VNNTLGNGSTVSKGYVSEDLAKNPTTGKDFTTDAVPSAVDYNLT AKKLWKEGLKEIGETKLNKLLSDDTDAGKKATEFIQGGWEKLPGLTV TNQNVPFKTRLQRSQNGNFDTVISAWNADFTDPISFLSLFTSDNSYN NGKYDSAAYDDAVKKAEGADANNKTARWADMVAAEKQLMNDQGV PIYQQASATLTRSNLKGIIYNTAGSNYNFKYMSVKE EQKLISEEDL (SEQ ID NO: 6)
Lp_3686 [UniProtKB - F9ULM7 (F9ULM7_LACPL)]	<u>MQAKYTYSIIGVV</u> <u>SALLTGCLGAVSNSSKTTSTADQNRTLGITIPGEP</u> MTTDPPTSJETNGGAVITQTGEGYRKNANNKIVAGVVEKKVKPTENK TRYTFTIRKNARWQNGTKITSQDFVTALRRQANPTTKSQNLDAISYIK NFDVAVNGKMAVNKLGVTAINKRTFSIKLSKVPYPMNYEFTSFYPLNT EAVQKYGSKLGSNSATTVANGAYVIKGWKGSNDSWYVVKNYWN AKNVKIKRIKVTVTKDDNTAQNLFNAGTVQVTNISGQYVKNANNSQ MTVTKTGRNNYIYFNSKKSVTANENFRHALSLVINQKTLAKDVLQDG SQAATNIVPKNYASDPTTGEDFVKEVGNLSPTNIKQAKQYWQKAQK ELGKQNVTLNFLCDDTDTEKKLAEYVQGVAEKYLKGVTKIVAVPHAT HVS RDFTGNFDLVTVGWGPDPDAQNFLDGMQSSNSINFTQWKDA KYDALMAKVSNTAKYTAAQRWYEEKQADRRLMKLDAVIPTYQASSA FLVSKDVGGLQWDEFSGTSSQLQYAYWKE EQKLISEEDL (SEQ ID NO: 7)

Pharmaceutical compositions

Disclosed herein is a pharmaceutical composition for intranasal administration, comprising a *Lactobacillus* bacterium engineered to produce and/or carry a drug.

- The bacterium may be mixed with a pharmaceutically-acceptable carrier or other vehicle. The carrier may be a liquid suitable, for example, for administration as nasal drops, nasal spray or nasal rinse, and includes water, saline or other aqueous or organic and preferably sterile solution. The carrier may be a solid, such as a powder, gel or ointment and may include inorganic fillers such as kaolin, bentonite, zinc oxide, and titanium oxide; viscosity modifiers, antioxidants, pH-adjusting agents, lyoprotectants and other

stability enhancing excipients, including sucrose, antioxidants, chelating agents; humectants such as glycerol, and propylene glycol; and other additives which may be incorporated as necessary and/or desired.

5 In general, a therapeutic amount of the active substance is delivered to the olfactory region. For single unit dose administration to the olfactory cavity, the volume administered is typically about 300 µl or less per nostril, preferably about 200 µl or less per nostril, more particularly about 100 µl or less per nostril.

Methods of treatment

10 The disclosure also provides methods of treating or preventing diseases and disorders of the central nervous system (CNS) through intranasal administration of bacteria engineered to produce and/or carry a therapeutic or prophylactic drug.

Disclosed herein is a method of treating a disease or disorder of the central nervous system (CNS) in a subject, the method comprising intranasally administering an effective amount of a *Lactobacillus* bacterium engineered to produce and/or carry a drug to the subject.

15 Also disclosed herein is a *Lactobacillus* bacterium engineered to produce and/or carry a drug, for use in treating a disease or disorder of the central nervous system (CNS) in a subject, wherein the bacterium is to be administered intranasally to the subject.

Disclosed herein is the use of a *Lactobacillus* bacterium engineered to produce and/or carry a drug in the manufacture of a medicament for treating a disease or disorder of the central nervous system (CNS) in a subject, wherein the bacterium is to be administered intranasally to the subject.

20 As used herein a "therapeutically effective amount" or "effective amount" is an amount that is non-toxic to the subject and sufficient to effect desired outcomes in a subject (i.e., achieve therapeutic efficacy). For purposes of this disclosure, a therapeutically effective amount of *Lactobacillus* bacteria is an amount of bacteria that is sufficient to produce or carry a drug in sufficient quantities to palliate, ameliorate, stabilise, 25 reverse, prevent, slow or delay the progression of a disease state. For example, the amount is typically that required to reach a specified or desired clinical endpoint, such as a decrease in the progression of the disorder, a lessening of the severity of the symptoms of the disorder and/or elimination of the disorder. This amount will vary depending on the time of administration, the route of administration, the duration of treatment, the specific composition used and the health of the patient as known in the art. The 30 skilled person will be able to determine the optimum dosage. A therapeutically effective amount can be administered in one or more administrations.

The terms "subject", "patient" or "host", used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not 35 restricted to, any member of the subphylum Chordata including primates (e.g., humans, monkeys and apes, and includes species of monkeys such from the genus *Macaca* (e.g., cynomolgus monkeys such as *Macaca fascicularis*, and/or rhesus monkeys (*Macaca mulatta*)) and baboon (*Papio ursinus*), as well

as marmosets (species from the genus *Callithrix*), squirrel monkeys (species from the genus *Saimiri*) and tamarins (species from the genus *Saguinus*), as well as species of apes such as chimpanzees (*Pan troglodytes*), rodents (e.g., mice rats, guinea pigs), lagomorphs (e.g., rabbits, hares), bovines (e.g., cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., pigs), equines (e.g., horses), canines (e.g., dogs), felines (e.g., cats), avians (e.g., chickens, turkeys, ducks, geese, companion birds such as canaries, budgerigars etc.), marine mammals (e.g., dolphins, whales), reptiles (snakes, frogs, lizards etc.), and fish. A preferred subject is a human in need of treatment for a disease or disorder of the CNS. However, it will be understood that the aforementioned terms do not imply that symptoms are present.

In one embodiment, the subject is a mammalian subject. In one embodiment, the mammalian subject is a human.

In some embodiments, the drug is releasable from the bacterium for delivery of the drug to the CNS to treat the disease or disorder. As described herein, the drug is a molecule that is neither encoded nor present within a non-engineered *Lactobacillus* bacterium.

A wide variety of diseases and conditions can be treated via intranasal delivery to the olfactory region. In particular, the methods of this disclosure are useful to treat any disease or disorder that has a therapeutic target in the olfactory region or the CNS, including but not limited to diseases and disorders of the CNS. Non-limiting examples of CNS diseases or disorders include acute head injury, spinal cord injury, stroke, ischemia, epilepsy, neurodegenerative diseases (e.g., Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Lewy body disease, Creutzfeldt-Jakob disease, dementia, etc.), infections (e.g., viral encephalitis, bacterial or viral meningitis, etc.), autoimmune disorders (e.g., multiple sclerosis, myasthenia gravis etc.), Duchenne dystrophy, leucodystrophies, psychiatric disorders (e.g., bipolar disorder, schizophrenia, etc.), mental illness (e.g., depression, anxiety disorder, post-traumatic stress disorder, etc.), migraines, neuropathy, acute pain, breakthrough pain, chronic pain, sleep disorders (e.g., insomnia, narcolepsy), cerebral palsy, anosmia. Other diseases or disorders include, but are not limited to, endocrine, metabolic or energy balance disorders that have a CNS component, such as obesity and diabetes. The methods herein may also be useful in post-surgical or post-treatment management of CNS injuries or diseases, to resolve side effects, complications and/or residual symptoms or otherwise to provide a palliative or stabilising effect during recovery.

In some embodiments, the disease or disorder of the CNS is a chronic disease or disorder. In some embodiments, the disease or disorder is a disease or disorder of the brain. In some embodiments, the disease or disorder is a metabolic disease or disorder. In one embodiment, the metabolic disease or disorder is obesity.

The drug may be any active substance that is capable of treating or preventing diseases or disorders in the olfactory region, in the brain, or in the central nervous system (CNS). Usually, but not always, the drug will be administered to treat or prevent diseases or disorders that have a therapeutic target in the olfactory region, brain or CNS. Substances that can be delivered include, but are not limited to, synthetic or natural organic pharmaceuticals, radiopharmaceuticals, synthetic or natural peptides, synthetic or natural polypeptides, antibodies, hormones, nucleic acids, sugars, carbohydrates, lipids.

Non-limiting examples of drugs that may be used for medical and/or prophylactic treatment include, e.g., antiviral substances; anti-prion substances; antibacterial substances, antifungal substances, antiparasitic substances, anti-inflammatory substances; and antidepressant substances. The drug may act as a neurotransmitter, neuromodulator, hormone, hormone releasing factor, hormone receptor agonist or antagonist, or neurotrophic factor. The drug may be an activator or inhibitor of a specific enzyme, an antioxidant, a free radical scavenger, a metal chelating agent, or an agent that alters the activity of ion channels in cells of the CNS. The drug may be any substance which is capable of acting as a stimulant, sedative, hypnotic, analgesic, anticonvulsant, antiemetic, anxiolytic, tranquillizer, cognition enhancer, agents preventing or healing amnesia, metabolic stimulator or inhibitor, appetite stimulator or inhibitor and/or narcotic antagonist or agonist. The drug may be any substance found to be deficient in conjunction with a CNS disease or disorder being treated or prevented, for example, a metabolite or a metabolic precursor.

In some embodiments, the drug is a small molecule, peptide, polypeptide or a combination thereof. In one embodiment, the drug is a peptide. In one embodiment, the drug is a polypeptide.

Without being bound by theory, the peptide or polypeptide may exert its effects by binding to, for example, cellular receptors in various regions of the brain. As one example, in order for α -melanocyte stimulating hormone (α -MSH) to exert its effect in body weight reduction, it binds to the melanocortin 4 receptor (MCR-4) on neurons in the hypothalamus. As a further example, in order for erythropoietin (EPO), active EPO fragments or EPO analogues to improve neurologic function after stroke or acute brain injury, it has to bind to neuronal receptors, e.g., on hippocampal cells, astrocytes, or similar cells.

A variety of therapeutic peptides or proteins, or biologically active portions thereof, may be delivered using the methods herein. For treating neurodegenerative diseases, the drug may be a neuroprotective or neurotrophic agent, e.g., leutenising hormone releasing hormone (LHRH) and agonists of LHRH, such as deslorelin; neurotrophic factors, such as those from the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5; the fibroblast growth factor family (FGFs), including acidic fibroblast growth factor and basic fibroblast growth factor; the neurokinin family, including ciliary neurotrophic factor, leukemia inhibitory factor, and cardiotrophin-1; the transforming growth factor family, including transforming growth factor- β 1–3 (TGF- β s), bone morphogenetic proteins (BMPs), growth/differentiation factors such as growth differentiation factors 5 to 15, glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, activins and persephin; the epidermal growth factor family, including epidermal growth factor, transforming growth factor- α and neuregulins; the insulin-like growth factor family, including insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2); the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagons superfamily, including PACAP-27, PACAP-38, glucagons, glucagons-like peptides such as GLP-1 and GLP-2, growth hormone releasing factor, vasoactive intestinal peptide (VIP), peptide histidine methionine, secreting and glucose-dependent insulinotropic polypeptide; and other neurotrophic factors, including activity-dependent neurotrophic factor and platelet-derived growth factors (PDGFs). Such agents are also

suitable for treating acute brain injury, chronic brain injury and neuropsychiatric disorders, such as depression.

Therapeutic polypeptides for treatment of eating disorders, such as for prevention of weight loss (anorexia) and weight gain (obesity), include melanocortin receptor (MCR) agonists and antagonists.

- 5 Suitable MCR agonists include α -melanocyte stimulating hormone (α -MSH) as well as β and γ -MSH, and derivatives thereof. Other peptides for obesity treatment include hormone peptide YY (PYY), leptin and ghrelin, leptin analogues and/or sensitisers, ciliary neurotrophic factor or analogues thereof, glucagon-like peptide-1 (GLP-1), insulin mimetics and/or sensitisers, and dopaminergic, noradrenergic and serotonergic agents.

- 10 Corresponding MCR antagonists regulating body weight homeostasis include endocannabinoid receptor antagonists, fatty acid synthesis receptor inhibitors, ghrelin antagonists, melanin-concentrating hormone receptor (MCHR) antagonists, PYY receptor antagonists and tyrosine phosphatase-1B inhibitors.

- 15 Therapeutic polypeptides for treatment of endocrine disorders, such as diabetes mellitus includes, for example, glucagon-like peptide 1 (GLP-1); peptides from the GLP-1 family, including pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), exendin-3 and exendin-4; and insulin-like growth factor (IGF-1), IGF binding protein 3 (IGFBP3) and insulin, and active fragments thereof.

- 20 In some embodiments, the bacterium is administered intranasally to the nasal cavity, preferably to the middle turbinate, superior turbinate or olfactory epithelium. These regions are typically located in the upper one-third portion of the nasal cavity.

In some embodiments, the bacterium is administered by nasal spray, nasal drops, nasal rinse, inhalation device, exhalation delivery system or nasal implant.

Definitions

- 25 The terms “treatment”, “treat”, or “treating” are used herein to refer to the reduction in severity of a disease or condition, the reduction in the duration of a disease; the amelioration or elimination of one or more symptoms associated with a disease or condition, or the provision of beneficial effect to a subject with a disease or condition. The term also encompasses prophylaxis of a disease or condition or its symptoms thereof. “Prophylaxis” is known in the art to mean decreasing or reducing the occurrence or severity of a particular disease outcome. For example, delaying progression of cancer in a subject.

- 30 As used herein, the term “subject” refers to a human or any non-human animal (e.g., mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). In many embodiments, a subject is a human being. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a disease. The term “subject” is used herein interchangeably with “individual” or “patient.” In some embodiments, the subject is human. A subject can be afflicted with or is susceptible to a disease or disorder but may or may not display symptoms of the disease or disorder.

In the context of the present invention, a drug is a molecule that can regulate biological processes and that is neither encoded nor present within a non-engineered *Lactobacillus* bacterium.

As used herein, a “small molecule drug” is an organic compound with a molecular weight less than 1,000 Daltons, that can regulate biological processes.

- 5 As used herein, “endogenous” refers to a molecule that is natively encoded and/or present within a host organism. For example, an OppA nucleic acid or polypeptide synthesised from the genome of a *Lactobacillus plantarum* WCFS1 bacteria is endogenous to said host bacteria.

- As used herein, “exogenous” refers to a molecule that is not natively encoded and/or present within a host organism. For example, an OppA nucleic acid or polypeptide that is not natively encoded and/or
10 present in host bacteria *Lactobacillus plantarum* WCFS1 is exogenous to said bacteria. As a further example, an OppA nucleic acid or polypeptide that is synthesised outside a *Lactobacillus plantarum* WCFS1 bacteria but introduced into the cytoplasm or onto the surface of said host bacteria is exogenous to said host bacteria. Any polypeptide produced by the host organism from a nucleic acid introduced into the host organism would be considered exogenous to said host organism.

- 15 As used herein, a “heterologous” sequence or gene or polypeptide refers to any sequence or gene or polypeptide that is natively encoded and/or present in an organism that is not the host organism. A heterologous sequence or gene or polypeptide can include a synthetically engineered sequence or gene or polypeptide.

- As used herein, a “homologous” sequence or gene or polypeptide refers to any sequence or gene or
20 polypeptide that is natively encoded and/or present in a host organism.

As used herein, a “commensal” organism refers to any organism that resides on the surface of a host organism or at mucosa without harming the health of the host organism. This includes, for example, *Lactobacillus plantarum* which is commensal to the human bladder tumour microenvironment.

- As used herein, a “probiotic” refers to a live organism that provides health benefits when administered to
25 a host organism. This includes, for example, engineered *Lactobacillus plantarum* as described herein for the treatment of bladder cancer in humans.

As used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (or).

- As used in this application, the singular form “a,” “an,” and “the” include plural references unless the
30 context clearly dictates otherwise. For example, the term “an agent” includes a plurality of agents, including mixtures thereof.

- Throughout this specification and the statements which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer
35 or step or group of integers or steps.

Throughout this specification and the statements which follow, unless the context requires otherwise, the phrase "consisting essentially of", and variations such as "consists essentially of" will be understood to indicate that the recited element(s) is/are essential i.e. necessary elements of the invention. The phrase allows for the presence of other non-recited elements which do not materially affect the characteristics of the invention but excludes additional unspecified elements which would affect the basic and novel characteristics of the method defined.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications, which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Unless otherwise defined, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

The features disclosed in the foregoing description, or in the following claims, or in the accompanying drawings, expressed in their specific forms or in terms of a means for performing the disclosed function, or a method or process for obtaining the disclosed results, as appropriate, may, separately, or in any combination of such features, be utilised for realising the invention in diverse forms thereof.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention.

For the avoidance of any doubt, any theoretical explanations provided herein are provided for the purposes of improving the understanding of a reader. The inventors do not wish to be bound by any of these theoretical explanations.

Any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word “comprise” and “include”, and variations such as “comprises”, “comprising”, and “including” will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

- 5 It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent “about,” it will be understood that the particular value forms another embodiment. The term “about” in relation to a numerical value is optional and means for example +/- 10%.
- 10

Examples

EXAMPLE 1 - *Lactobacillus* strains localise to the olfactory epithelium (OE)

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The human nasal microbiome has previously been studied. Among the commensal microbes in the nose, the genera *Lactobacillus*, *Streptococcus*, and *Escherichia* contain probiotic strains suitable for otolaryngology applications. To identify a chassis bacteria that could localise in the OE, we tested eight probiotic strains for binding efficacy to heparan sulphate (NaHS). These strains are *Lactobacillus plantarum* WCFS1 (Lp), *Lactobacillus sakei* DSM 20017, *Lactobacillus casei* DSM 20011, *Lactobacillus delbrueckii* DSM 20072, *Lactobacillus rhamnosus* GG, *Streptococcus salivarius* K12, *Streptococcus thermophilus* ATCC 19258, and *Escherichia coli* Nissle 1917. Specifically, the *Lactobacillus* strains used are among the five most abundant *Lactobacillus* species in the nasal cavity. Additionally, the two *Streptococcus* strains and *E. coli* Nissle have been investigated for use as oral and nasal probiotics.

20

- 25 The olfactory epithelium (OE) uniquely features an apical distribution of NaHS, distinguishing it from other nasal epithelia. The apical NaHS provides a binding site for a variety of substance, enabling for example viruses such as the herpes simplex virus and the SARS-CoV-2 virus to penetrate the OE and potentially access the CNS. The murine myeloma NS0 cell line was previously reported to express NaHS on its cell surface and has been used to investigate the interaction between virus proteins and the OE. Building on this, the NS0 cell line was used to examine bacterial binding to NaHS and, by extension, the OE (Figure 2a). As shown in Figure 2b, the *Lactobacillus* strains exhibited significantly higher binding efficacy than other strains, among which Lp displayed the highest binding efficacy towards NS0 cells. The incubation of the FITC-labeled Lp strain with NS0 cells resulted in over 24% of NS0 cells binding to the bacteria (Figure 2b). Based on these findings, it was hypothesised that Lp has an affinity for NaHS, positioning it as a potential candidate strain for OE attachment.
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- 35

Immunoprecipitation assay was performed to identify potential NaHS binding proteins in Lp located on its bacterial membrane (Figure 3a and 3b). Through a pull-down using immobilised biotinylated NaHS, a

unique 63 kDa NaHS-binding protein was identified (Figure 3c). MALDI-TOF (MS) analysis of this protein band identified it as Lp_0783, a member of the OppA family of proteins, which has been previously reported as heparan sulphate binding proteins. However, Lp_0783 was recovered with a low protein sequence coverage (6.7%), possibly due to interference from OppA-homologues present in Lp, which have similar binding affinity and protein sizes.

To fully characterise the binding capacity of OppAs to NaHS, all seven OppA proteins from Lp were recombinantly expressed and incubated with two heparan sulphate-producing cell lines: CHO-K1 and NS0 cells. CHO-K1 cells express both N-acetyl and N-sulpho heparan sulphate, and NS0 cells express only NaHS. As shown in Figure 2b and 2c, the OppA proteins demonstrated different binding strengths to the two cell lines, exhibiting a reverse trend. Three proteins, Lp_0092, Lp_0783, and Lp_3686, exhibited the lowest binding capacity among the OppAs to CHO-K1 cells (Figure 3d), but displayed significantly stronger binding to NS0 cells (Figure 3e). This finding suggests that Lp 0092, Lp 0783 and Lp_3686 preferentially binds to NaHS and could potentially facilitate bacterial localisation to the OE.

To assess the capability of Lp to target the OE, the Lp strain was metabolically labelled with fluorescein isothiocyanate (FITC) and administered intranasally to mice (Figure 4a). Post-administration, the snout and brain were harvested and visualised for the localization of Lp in relation to the nasal epithelia (Figure 4b). From both dorsal (Figure 4c) and lateral (Figure 4d) perspectives, the Lp strain was primarily localised in the OE, adjacent to the OB, 24 hours post-administration (outlined by white boxes). However, by 48 and 72 hours, there was a noticeable reduction in bacterial presence within the OE, as indicated by diminished fluorescence intensity.

To investigate the involvement of OppA proteins in OE localisation, Lp_0783 and Lp_0092 were deleted from the genome of Lp, yielding the $\Delta O2$. Intranasally-administered FITC-labeled $\Delta O2$ in mice showed that the absence of the two OppA proteins significantly reduced the localisation of the $\Delta O2$ strain in the OE, as evidenced by the reduced fluorescence intensity (Figure 4c & d). The radiant efficiency of the nasal epithelia was then quantified based on their fluorescence intensity. Figure 4e shows that there is no significant radiant efficiency difference in the RE of mice treated with PBS, Lp, or $\Delta O2$. However, Figure 4f shows that the deletion of OppA led to a nearly 50% radiant efficiency reduction in the OE, indicating a significant reduction in the OE-targeting capability of the $\Delta O2$ strain. These results suggest that Lp can specifically localise in the OE upon intranasal administration, and the NaHS-binding OppA proteins enhance this localisation.

FITC was then used as a tracer molecule to track the transport of potential therapeutic payloads from Lp to the OE. To increase the visibility of FITC in the nasal cavity, FITC-labeled Lp were administered intranasally to mice daily for 3, 7, and 14 days. The mice were euthanised 24 hours after the last administration, and their snouts and brains were harvested, sectioned, and imaged. As shown in Figure 5a and 5b, following 3 days of bacterial administration, FITC started accumulating in the lamina propria beneath the OE. After sustained administration for 7 and 14 days, FITC permeated the intercellular spaces of the lamina propria and diffused towards the nasal septum below, manifesting intense

fluorescence signals in both areas (Figure 5a and 5b). The specific accumulation of FITC in the lamina propria and adjacent nasal septum suggests the OE as the main release point for FITC by the Lp strain.

As with Figure 5, FITC-labelled Lp was intranasally administered intranasally to mice daily for 3, 7, and 14 days (Figure 6). The mice were euthanised 24 hours after the last administration, and their snouts and
5 brains were harvested, sectioned to reveal different levels of nasal epithelia (Figure 6a), and imaged. The anti-OMP (olfactory marker protein) antibody was used for staining olfactory marker proteins in the olfactory neurons present in the OE and olfactory nerve bundles in the lamina propria. FITC or FITC labelled Lp were absent from the RE of the treated mice. Deeper sections (Figure 6) revealed that FITC accumulates in the lamina propria beneath the OE after 3 days of daily bacterial administration.

10 Biomolecules in the OE profess to the lamina propria and subsequently to the brain through various pathways including intracellular transport via olfactory neurons, transcellular transport through supporting cells, or paracellular diffusion (Figure 6b). The pronounced presence of FITC at the interface between the OE and the lamina propria suggests the movement of FITC molecules through the OE to access the lamina propria (Figure 6b, 6d & 6e).

15 Harvesting of the lungs, liver, spleen, kidneys, stomach and intestine from mice subjected to 14 days of treatment was used to determine the distribution of Lp and FITC outside the nasal cavity. FITC was not detected in the lungs, livers, spleens or hearts suggesting these organs are not the primary site for bacterial localisation or FITC distribution. Homogenisation and CFU counts of these organs indicated no translocation of live bacteria to these organs. FITC in faeces pellets in the intestine suggests ingestion
20 and elimination of excessive bacteria from the nasal cavity. These bacteria might account for the observed decrease in bacterial numbers in the OE 48 hours following intranasal administration. The FITC released in the gut might also contribute to the FITC found in the circulation and the kidneys.

The accumulation of FITC in the lamina propria is crucial in enabling its translocation to the OB in the brain through the olfactory nerves. As shown in Figure 6b, the lamina propria is a thin layer of areolar
25 connective tissue that provides access to various structures, including blood vessels, lymphatic vessels, and, importantly, olfactory nerves. The basal extensions of the olfactory neurons in the OE form olfactory nerve bundles (ONBs), which ascend through the lamina propria, traverse the cribriform plate, and ultimately reach the OB in the brain (Figure 6b). These ONBs are encased by ensheathing cells that provide a protected conduit for axonal transmission over extended distances. Additionally, the olfactory
30 ensheathing cells are covered by olfactory nerve fibroblasts, constituting the perineurium that surrounds the olfactory nerves leading to the OB. Through the perineurial space between the olfactory ensheathing and olfactory nerve fibroblasts, substances within the lamina propria can translocate further into the OB (Figure 6b). Therefore, the olfactory nerves serve as a critical link between the nasal cavity and the central nervous system (CNS), forming the olfactory pathway; their axons penetrate the cribriform plate,
35 which separates the nose from the brain. Intranasally delivered substances like FITC can disperse through this the perineurial space, allowing them to access the OB and potentially other parts of the brain via the subarachnoid space.

To investigate the possible transportation of FITC from the OE to the OB, we analyzed its translocation from the lamina propria to contiguous structures. As shown in Figures 6d and 6e, the diffusion of FITC extends to adjacent tissues in the lamina propria following 14 days of administration. As the sections approach the OB, the OE layer becomes thicker, with a growing number of olfactory neurons in the OE and increasing size of ONBs in the corresponding lamina propria. FITC was also found to accumulate at the periphery of the ONBs (Figure 6d & 6e), with increasing predominance as the sections deepened, potentially providing access to the CNS.

Such diffusion and accumulation of FITC in the lamina propria is already evident in the nascent OE following 3 days of intranasal administration (Figure 7a). However, examination of deeper sections of the OE revealed the accumulation of FITC in the periphery of the cribriform plate and the infusion of FITC into the OB through the perineural space following 7- and 14-days administration of Lp (Figure 7b). *In vivo* imaging using IVIS confirmed the progressive accumulation of FITC in the OB following intranasal administration on the 7th and 14th day (Figure 7c & 7d), which is consistent with the immunofluorescence results. Additionally, lateral view imaging of the brain reveals fluorescent signals in other regions, indicating a broader distribution of FITC within the brain (Figure 7e & 7f). These findings substantiate that by targeting the OE, Lp can effectively deliver the payload FITC to the brain via the olfactory pathway and delivers FITC to the forebrain rather than the trigeminal pathway which targets the brainstem.

Compared to the mice that received intranasal Lp, those treated with intravenous FITC showed relatively higher FITC accumulation in the kidneys but no FITC accumulation in the OBs or brains in general. These findings confirm that by targeting the OE, intranasally administered Lp can effectively deliver the payload FITC to the brain via the olfactory pathway.

EXAMPLE 2: Nose-to-brain drug delivery using intranasally delivered engineered *Lactobacillus* strains

To evaluate the efficacy of intranasal therapeutic delivery using obesity as a model disease, Lp was engineered to express and secrete three appetite-regulating hormones from the leptin-melanocortin pathway: Leptin, α -MSH, and BDNF (Figure 8a). Protein secretion was engineered by utilising a native Lp secretion signal peptide from Lp_3050. The secreted Leptin and BDNF were detected on western blots via the detection of N-terminal 6x His tags (SEQ ID NO: 8) (Figure 8b and 8c). Lp culture and protein expression in MRS media yielded approximately 20 mg/L of leptin and BDNF. Due to its small molecular weight (1.6kDa), the secretion of α -MSH was not visualised on the western blot; however, it was confirmed using an α -MSH ELISA kit, which measured its concentration at approximately 23 pg/ml in the culture media (Figure 8d). When co-cultured with human nasal cells, the engineered Lp strain secreted up to 350 ng/L of Leptin, 160 ng/L of α -MSH, and 15 ng/L of BDNF in the media.

To assess the bioactivity of the secreted recombinant hormones, human embryonic kidney 293 (HEK) cells expressing either the leptin or tropomyosin receptor kinase B (TrkB) receptor were exposed to leptin and BDNF purified from Lp cultures. The introduction of Lp-secreted Leptin resulted in the dose-dependent phosphorylation of the transcription factor – signal transducer and activator of transcription 3

(Stat3), suggesting activation of the leptin receptor and verifying the bioactivity of the recombinant Leptin (Figure 8b). Similarly, the supplementation of recombinant BDNF also led to the phosphorylation of the TrkB receptor in transfected HEK cells (Figure 8c). The bioactivity of the secreted α -MSH was evaluated by introducing the Lp culture supernatant to the mouse hypothalamus Neuro2A cells. The addition of the supernatant led to a significant increase of cyclic adenosine monophosphate (cAMP) in Neuro2A cells confirming that the recombinant α -MSH activated the melanocortin-4 receptors (MC4R). This suggests its potential efficacy in inducing satiety in mice (Figure 8d).

To assess the engineered Lp's ability to produce and deliver appetite-regulating hormones, we employed an in vitro intranasal delivery model using differentiated RPMI 2650 nasal squamous cancer cells.

Characterized by TEER values closely resembling the nasal epithelium this model has been extensively used to predict the permeability of various intranasally-administered therapeutics. The RPMI 2650 cells were used to form a monolayer in the transwell, simulating the OE. Subsequently, the Lp strains were introduced to the transwell to deliver the therapeutic payloads or FITC. The RPMI 2650 cells underwent differentiation at an air-liquid interface, and co-culturing commenced once the TEER of the cells attained $30 \Omega \cdot \text{cm}^2$. Throughout the 72-hour co-culture, the integrity and permeability of the RPMI 2650 monolayer remained unaffected by Lp, as validated by consistent TEER measurements of the monolayer.

Initially, FITC-labeled Lp was incorporated to the in vitro model to simulate the intranasal delivery of FITC. As shown in Figure 9a, the release of FITC from Lp led to a gradual increase of FITC in the basal compartment over 72 hours of co-culture. This gradual FITC translocation and accumulation align with in vivo FITC accumulation patterns in the lamina propria, confirming the successful replication of the in vitro intranasal delivery model. We then tested the delivery of Leptin, α -MSH and BDNF by the engineered Lp strains in this model. All three hormones were fused with a HiBiT tag at the C-terminus to measure the concentration of hormones in the transwells and basal compartment.

As shown in Figure 9a and 9b, the total concentration of the proteins peaked at 24 hours and rapidly declined over time at 48 and 72 hours. The recombinant hormones were expressed and secreted in a growth-dependent manner and reached the maximum concentration near the end of exponential growth of Lp around 24 hours (Klumpp and Hwa, 2008). After that, the degradation of the hormones was anticipated, owing to their short half-lives under physiological conditions. Notably, the peak concentration of secreted Leptin were much higher than the FITC released by Lp (Figure 9a). Between the groups, the transference of Leptin and α -MSH emerged as notably more efficient than FITC (Figure 9b). By the 24-hour mark, roughly 40% of both Leptin and α -MSH were discernible in the basal compartment, nearly double the concentration of FITC in the same zone (Figure 9b). The delivery efficiency of BDNF by Lp was also comparable to that of FITC, as indicated by their similar basal compartment distribution after a 24-hours co-culture (Figure 9b). Compared to Leptin

and α -MSH, BDNF showed a lower transference efficiency. This is likely due to its high stickiness and the increased molecular size resulting from its dimerization.

Three engineered Lp strains were subsequently introduced intranasally into mice, each strain secreting Leptin, α -MSH or BDNF. Peptide delivery within the OE was observed. As shown in Figure 9c and 9d, 24

hours post intranasal administration of the engineered Lp strains, all three recombinant hormones were observed predominantly in the supporting cells in the OE. This distribution contrasts with the paracellular transport pattern of FITC, suggesting that the secreted hormones might be transported intracellularly by the supporting cells to access deeper structures beneath the OE. Notably, 48 hours after administration, both secreted Leptin and BDNF remained visible in the supporting cells, while α -MSH in the OE became undetectable (Figure 9c). This may be attributed to the shorter half-life of α -MSH, suggesting the rapid degradation and limited accumulation of α -MSH in the OE. Taken together, these results suggest the potential of Lp as a robust delivery system for secreting and intranasally delivering therapeutic agents to the OE.

To assess the in vivo efficacy of our engineered Lp, we initially administered different doses of wildtype Lp intranasally to determine a regimen that wouldn't result in weight loss due to nasal obstructions. A moderate dose of involving 5×10^7 CFU of bacteria administered alternately per nostril daily, significantly curtailed nasal polyp formation and did not affect weight growth compared to PBS-treated mice.

To assess the potential of the bacteria in combating obesity, engineered Lp strains were given to C57BL/6 mice on a high-fat diet, following the moderate regimen (Figure 10a). Over the eight-week treatment, mice treated with Leptin-secreting Lp exhibited reduced weight gain after three weeks, compared to those given PBS or wildtype bacteria (Figure 10b). After Leptin, mice treated with Lp secreting α -MSH and BDNF showed reduced weight gain within four and five weeks, respectively (Figure 10b). However, only leptin- and α -MSH-secreting Lp significantly decreased daily food intake (Figure 10c). The observed reduction in weight gain due to BDNF might be partially linked to its established role in enhancing metabolic activity.

The engineered microbes also showed enhanced glucose metabolism, as evidenced by significantly lower fasting glucose levels in the mice receiving hormone-expressing Lp (Figure 10d). Moreover, mice in the Leptin and α -MSH group showed better maintenance of blood glucose in the intraperitoneal glucose tolerance test (IPGTT), marked by a significantly faster restoration of glucose level following a glucose challenge (Figure 10e). Furthermore, these mice displayed improved lipid metabolism, shown by significantly reduced body fat ratios in subcutaneous (Figure 10f), peri-gonadal (Figure 10g), and perirenal regions (Figure 10h). The hepatic triacylglycerol levels also decreased in the α -MSH and BDNF-treated mice (Figure 10i). Correspondingly, the administration of the engineered microbes prevented liver micro-steatosis, a condition that was evident only in mice treated with PBS and those given the empty vector control (Figure 10j). We subsequently formulated a treatment regimen, integrating all three Lp strains (Lp-Mix), to probe possible synergistic hormone effects. Here, Lp strains, expressing individual hormones, were mixed in equal parts, adjusting each strain's dosage to a third of the CFU used in singular groups, thus ensuring consistent hormone dosages. The treatment using a mix of strains resulted in only modest improvements in weight gain, food intake, glucose regulation, and lipid profiles. This outcome is likely attributed to the lower dosages of individual hormones administered. Moreover, the anticipated synergistic effect was not observed in the combination treatment, possibly due to the

overlapping mechanisms of action of the individual hormones, which act via the leptin-melanocortin pathway. Taken together, the intranasal

administration of the engineered Lp strains significantly reduced food intake, maintained body weight, and improved glucose and lipid metabolism in mice on a high-fat diet.

5 Among the three engineered Lp strains, Lp secreting Leptin led to the lowest body weight gain, daily food intake and a better glucose response in mice. Therefore, to demonstrate the advantages of the engineered Lp in combating obesity, engineered Lp secreting Leptin or recombinant Leptin (r-Lep) were administered to C57BL/6 mice on a high-fat diet, following a daily or alternate-day dosing regimen for 8 weeks (Figure 11a). In mice receiving recombinant Leptin, the peptide was undetectable in the OE 24 or
10 48 hours post intranasal administration (Figure 11b). In contrast, intranasal administration of engineered Lp significantly extended the presence of Leptin in the OE (Figures 8c and 8d).

Over the 8-week treatment period, mice that received engineered Lp exhibited decreased total body weight, reduced body weight gain (Figure 11c), and lower food intake (Figure 8d), regardless of the dosing frequency. Similar reductions in body weight gain and food intake were observed in mice receiving
15 daily administration of recombinant Leptin; however, this effect was completely abolished in mice receiving Leptin on alternative days. Mice receiving lower doses of Leptin showed significantly higher body weight, body weight gain (Figure 11c), and food intake (Figure 11d) compared to all other treatment groups. Additionally, intranasal administration of engineered Lp, whether given daily or every other day, led to significantly improved glucose metabolism, as evidenced by lower fasting glucose levels (Figure
20 11e) and faster recovery of blood glucose levels following intraperitoneal glucose challenge (Figure 11f). Notably, all treatments resulted in reduced accumulation of adipose tissue, with Leptin treatment accounting for 32.8% to 48.8% reduction in mass across three fat pads, and engineered Lp treatment showing 45.1% to 58.5% reduction (Figure 11g, 11h, and 11i). In summary, intranasal administration of Leptin-secreting Lp significantly prolonged Leptin presence in the OE, providing sustained suppression of
25 appetite, maintenance of body weight, and improvement in glucose and lipid metabolism compared to recombinant Leptin in lean mice on a high-fat diet.

In conclusion, prior investigations have explored the intranasal administration of Leptin, α -MSH and BDNF, demonstrating elevated concentrations of these hormones in the hypothalamus when compared to systemic delivery methods. However, the existing delivery methods of intranasal administrations have
30 many limitations. Firstly, the existing delivery methods do not specifically target the OE, leading to suboptimal efficacy and potential loss of therapeutic hormones within the RE. Secondly, these hormones display short physiological half-lives, typically spanning from a few minutes up to two hours. Consequently, maintaining therapeutically-relevant CNS hormone levels necessitates frequent dosing at intervals of a few hours, which presents a significant barrier to their broader clinical translation.

35 The data presented herein showed intranasal administration of Lp discharged the tracer molecule FITC distinctly within the OE. This process resulted in the diffusion and eventual accumulation of FITC in the lamina propria beneath the OE, and finally in the OB within the brain. This shows the selectivity of Lp towards the OE and the delivery route of the payload molecules in the olfactory pathway.

The efficacy of intranasal delivery for therapeutic applications was shown with obesity serving as the model condition. A Lp strain was modified to produce and transport three hormones that play crucial roles in the Leptin-melanocortin pathway. Recombinant expression by live bacteria localized at the OE, showed continuous release of these appetite-regulating hormones within the OE. The bioavailability of these hormones was significantly prolonged, from merely a few hours to over 24 hours. α -MSH was detectable in the olfactory epithelium (OE) for up to 24 hours, while Leptin and BDNF levels persisted for at least 48 hours following the final Lp administration. During an eight-week treatment period, mice receiving daily doses of the hormone-producing Lp exhibited significant reductions in weight gain and demonstrated improvements in both glucose and lipid metabolism.

- 10 The data also shows that intranasally administered recombinant Leptin could not be detected 24 hours post-administration, whereas engineered Lp extended the presence of Leptin to at least 48 hours. When administered on a low-frequency regimen, the intranasal administration of the engineered Lp secreting Leptin showed a marked improvement in preventing obesity in mice compared to recombinant Leptin, demonstrating better appetite suppression, maintenance of body weight, and enhanced glucose metabolism. The present invention therefore provides advantageous intranasal delivery of Lp to target the OE for sustained therapeutic efficacy within the brain.

- 20 The present invention using Lp for intranasal delivery has the advantages of being a precise method to target the OE and to provide continuous release of the therapeutics in the OE for sustained therapeutic action in the brain. Commensal Lp bacteria can potentially deliver a diverse array of therapeutic molecules, including small metabolites (FITC), peptides (α -MSH), and recombinant proteins (Leptin and BDNF). These results suggest that CNS drug delivery using engineered *Lactobacillus* can facilitate proactive modulation of brain functions and potentially provide therapeutic interventions for a spectrum of CNS and neurological disorders (e.g., brain diseases and neurodegenerative diseases) and metabolic disorders.

- 25 It will be appreciated that many further modifications and permutations of various aspects of the described embodiments are possible. Accordingly, the described aspects are intended to embrace all such alterations, modifications, and variations that fall within the spirit and scope of the appended statements.

30 **References**

A number of publications are cited above in order to more fully describe and disclose the invention and the state of the art to which the invention pertains. Full citations for these references are provided below. The entirety of each of these references is incorporated herein.

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Claims:

1. An engineered *Lactobacillus* bacterium:
 - a) comprising an expression construct encoding a drug that is releasable from the bacterium and/or
 - b) carrying a drug that is releasable from the bacterium,
- 5 wherein said drug can act upon the central nervous system (CNS) after passing the blood-brain-barrier (BBB).
2. A method of delivering a drug to the CNS of a subject, the method comprising intranasally administering a *Lactobacillus* bacterium engineered to produce and/or carry the drug to the subject,
- 10 wherein the drug is releasable from the bacterium for delivery to the CNS.
3. The engineered *Lactobacillus* bacterium of claim 1, or the method of claim 2, wherein the *Lactobacillus* bacterium is *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus casei*, *Lactobacillus delbrueckii* or *Lactobacillus rhamnosus*.
- 15 4. The engineered *Lactobacillus* bacterium of claim 3, or the method of claim 3, wherein the *Lactobacillus* bacterium is *Lactobacillus plantarum* (Lp).
5. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 or 4, or the method of any one of
- 20 claims 2 to 4, wherein the bacterium is engineered to secrete the drug.
6. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 to 5, or the method of any one of claims 2 to 5, wherein the bacterium is administered intranasally to the nasal cavity.
- 25 7. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 to 6, or the method of any one of claims 2 to 6, wherein the bacterium is administered by nasal spray, nasal drops, nasal rinse, inhalation device, exhalation delivery system or nasal implant.
8. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 to 6, or the method of any one of
- 30 claims 2 to 7, wherein the bacterium comprises an OppA polypeptide on a surface of the bacterium.
9. The engineered *Lactobacillus* bacterium of claim 8, or the method of claim 8, wherein the OppA polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to an amino acid sequence as set forth in SEQ ID
- 35 NO: 1–7.
10. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 to 9, or the method of any one of claims 2 to 9, wherein the drug is a small molecule, peptide, polypeptide or a combination thereof.

11. The engineered *Lactobacillus* bacterium of claim 10, or the method of claim 10, wherein the drug is a peptide and wherein the peptide is a hormone, optionally wherein the hormone is any one of Leptin, α -MSH, BDNF or any combination thereof.
- 5 12. The engineered *Lactobacillus* bacterium of claim 11, or the method of claim 11, wherein the hormone is Leptin.
13. The engineered *Lactobacillus* bacterium of any one of claims 1, 3 to 12, or the method of any one of claims 2 to 12, wherein the bacterium localises to the olfactory epithelium (OE) of the subject.
- 10 14. The engineered *Lactobacillus* bacterium of claim 13, or the method of claim 13, wherein the bacterium binds to apical heparan sulphate (NaHS) on neuronal cilia in the subject.
- 15 15. The engineered *Lactobacillus* bacterium of claim 14, or the method of claim 14, wherein upon release of the drug, the drug traverses the OE and reaches the olfactory bulb (OB) in the subject.
16. The engineered *Lactobacillus* bacterium of any one of claims 14 to 15, or the method of any one of claims 14 to 15, wherein the drug traverses the lamina propria (LP) in the subject.
- 20 17. The engineered *Lactobacillus* bacterium of any one of claims 14 to 16, or the method of any one of claims 14 to 16, wherein the drug traverses the olfactory nerve bundle (ONB) in the subject.
18. The engineered *Lactobacillus* bacterium of any one of claims 14 to 17, or the method of claims 14 to 17, wherein the drug traverses the cribriform plate (CP) in the subject.
- 25 19. The engineered *Lactobacillus* bacterium of any one of claims 14 to 18, or the method of any one of claims 14 to 18, wherein the drug traverses through the perineural space in the subject.
20. The engineered *Lactobacillus* bacterium of any one of claims 14 to 19, or the method of any one of claims 14 to 19, wherein the drug diffuses from the OB to the rest of the brain in the subject.
- 30 21. The engineered *Lactobacillus* bacterium of any of claims 1, 3 to 20, or the method of any of claims 2 to 20, comprising administration of the drug in:
- 35 (a) a daily-dosing regimen, or
- (b) an alternate-day dosing regimen.
22. A method of treating a disease or disorder of the central nervous system (CNS) in a subject, the method comprising intranasally administering an effective amount of a *Lactobacillus* bacterium engineered to produce and/or carry a drug to the subject.
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23. The method of claim 22, wherein the drug is releasable from the bacterium for delivery of the drug to the CNS to treat the disease or disorder.
24. The method of any one of claims 22 or 23, wherein the disease or disorder is a chronic disease or disorder.
25. The method of any one of claims 22 to 24, wherein the disease or disorder is a disease or disorder of the brain.
26. The method of any one of claims 22 to 25, wherein the disease or disorder is a metabolic disease or disorder.
27. The method of claim 26, wherein the metabolic disease or disorder is obesity.
28. A *Lactobacillus* bacterium engineered to produce and/or carry a drug, for use in treating a disease or disorder of the central nervous system (CNS) in a subject, wherein an effective amount of the bacterium is to be administered intranasally to the subject.
29. The engineered *Lactobacillus* bacterium for the use of claim 28, wherein the *Lactobacillus* bacterium is any one of claims 1, or 3 to 21.
30. Use of a *Lactobacillus* bacterium engineered to produce and/or carry a drug in the manufacture of a medicament for treating a disease or disorder of the central nervous system (CNS) in a subject, wherein an effective amount of the bacterium is to be administered intranasally to the subject.
31. The use of claim 29, wherein the *Lactobacillus* bacterium is any of claims 1, or 3 to 21.
32. A pharmaceutical composition for intranasal administration, comprising a *Lactobacillus* bacterium engineered to produce and/or carry a drug.
33. The pharmaceutical composition of claim 32, comprising a *Lactobacillus* bacterium of any of claims 1, or 3 to 21.
34. The pharmaceutical composition of claim 32 or 33, comprising one or more *Lactobacillus* bacteria.
35. The pharmaceutical composition of claim 32, comprising a first bacterium, a second bacterium and a third bacterium according to any one of claims 1, or 3 to 21, wherein the first bacterium is engineered to produce and/or carry a payload comprising Leptin and the second bacterium is engineered to produce and/or carry a payload comprising α -MSH, and the third bacterium is engineered to produce and/or carry a payload comprising α -MSH.

36. A kit comprising the bacterium of any of claims 1 to 21.
37. The kit of claim 36, comprising a first container containing a first composition according to any one of claims 32 to 35 and a second container containing a second composition according to any one of
5 claims 32 to 35, wherein the drug expressed and/or carried by the bacterium of the first composition comprises a different drug to the drug expressed and/or carried by the bacterium of the second composition.
38. The kit of claim 37, further comprising a third container containing a third composition according to
10 any one of claims 32 to 35, wherein the drug expressed and/or carried by the bacterium of the third composition comprises a different drug to the drug expressed and/or carried by the bacterium of the first and second compositions.