

**METHODS OF TREATING CANCER USING ENGINEERED LACTOBACILLUS
BACTERIA**

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

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 008686016, and is 12,515 bytes in size.

Field of the Invention

The present invention relates, in general terms, to methods of treating cancer and more specifically to methods of treating cancer using *Lactobacillus* bacteria which have been engineered to display a capture domain for binding a drug.

Background

Chemotherapy plays a critical role in the treatment of various cancers, especially in reducing distant metastasis. However, chemotherapy regimens are known for their poor bioavailability at certain tumour sites, substantial systemic toxicity and low patient tolerance. For many patients, prolonged chemotherapy is unbearable and as a result, distant metastasis has become the leading cause of treatment failure in many cancers. There is thus a need for drug delivery vectors capable of targeted delivery and localised release of anti-cancer therapeutics to improve the effectiveness of cancer treatment and to allow de-escalation of chemotherapy regimens.

Prodrugs are molecules with little pharmacological activity that can be converted to active native drugs *in vivo* by chemical or enzymatic reactions. Higher selectivity of prodrugs can be achieved by leveraging physiological conditions unique to the tumor microenvironment (TME). In this case, cytotoxic drugs are modified by chemical groups that are responsive to TME cues and are released when they reach tumors in a site-specific manner. This is achieved by the recognition of tumor-specific markers, such as antigens or receptors targeted by antibody conjugated drugs (ADCs). To date, 14 ADCs have received market approval from the US Food and Drug Administration (FDA) for cancer treatment, marking a significant advancement for targeted cancer therapy (Fu et al., 2022).

However, current prodrug strategies have limited target specificity. For instance, TME cues are not clearly distinguishable from normal tissue, which results in significant off-target effects in prodrugs. Further, the macromolecular nature of conjugated tumor-targeting carriers often complicates the pharmacokinetic

profiles of the prodrugs in the circulation, affecting their biodistribution, metabolism and clearance. In the case of ADCs, conjugating the antibody carriers to the native drugs substantially increases the size of the molecule, which impairs the penetration and bioavailability of the drugs in the tumors (Xu et al., 2015).

The longer half-life of the carrier antibodies also prolongs the clearance of payload drugs in the body,

5 damaging liver and kidney functions (Khera et al., 2018). In addition to systemic toxicity caused by chemotherapy, systemic administration of ADCs can introduce toxicity from the antibody components, inducing immune responses and causing severe secondary injuries that lead to nephrotoxicity in patients (Mecklenburg et al., 2018). Several preclinical studies have also revealed similar complications and side effects for various nanomaterial carriers (Cheng et al., 2021).

10 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.

Summary of the Invention

15 To overcome the limitation stemming from confined treatment specificity and associated complications, the inventors discovered and harnessed the intrinsic interactions between bacteria and cancer cells within the tumor microbiome for the precise administration of prodrugs. The inventors hypothesised that they could pinpoint commensal microbes endowed with cancer-binding capabilities and engineer them to precisely deliver prodrugs to specific cancer sites. The payload prodrugs can be conjugated to the bacterial vector through chemical linkers to ensure the site-specific release of the native drugs.

20 Commensal microbes were identified and characterised with the ability to bind to cancer cells and subsequently engineered to enable the precise delivery of chemotherapy agents to cancer sites, using nasopharyngeal carcinoma (NPC) as a representative model for cancer. NPC is the most common head and neck cancer and has a high incidence rate in Southern China, Southeast Asia, and North Africa, affecting over 133,000 people and causing over 80,000 deaths in 2020 alone (Tang et al., 2016). By 25 2040, it is projected that the global number of NPC cases and deaths will rise to approximately 179,000 and 114,000, respectively (Zhang et al., 2023). Chemotherapy in NPC is associated with substantial toxicity and difficulties in administration, making prolonged chemotherapy intolerable for patients (McDowell et al., 2020; Wang et al., 2019).

30 In one aspect, disclosed herein is a method of treating a cancer in a subject, the method comprising administering an effective amount of engineered *Lactobacillus* bacteria carrying a drug to the subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium. In some embodiments, the bacteria are derived from a cancer tissue or a tissue where the cancer can occur. In preferred embodiments, the bacteria are of the species *Lactobacillus plantarum* (Lp). In some 35 embodiments, the Lp is of the strain WCFS1. 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 attached to the cell wall or cell membrane of the bacteria. The bacteria may contain one or more OppA polypeptides. In some
5 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.

10 In some embodiments, the drug comprises a tag that is capable of binding to the capture domain. In some embodiments, the capture domain is a biotin-binding polypeptide, and the tag is biotin. In some embodiments, biotin-binding polypeptide is streptavidin. In some embodiments, the capture domain comprises an anchor domain for anchoring to the bacterial surface. In some embodiments, the anchor domain is a bacterial transmembrane domain. In some embodiments, the bacterial transmembrane domain comprises an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%,
15 98%, 99% or 100% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 8.

In some embodiments, the drug is a prodrug comprising a cleavable entity. In some embodiments, the prodrug is activated in a vicinity of a cancer cell or tissue through cleavage of the cleavable entity. In some embodiments, cleavable entity is a redox-sensitive entity. In some embodiments, the bacteria is administered mucosally, peri-tumourally or intra-tumourally.

20 In some embodiments, the cancer is a carcinoma. In some embodiments, the cancer is a nasopharyngeal, oral, lung, bladder, gastric, colorectal, skin, breast or ovarian cancer. In preferred embodiments, the cancer is a nasopharyngeal cancer.

In a second aspect, disclosed herein is an engineered *Lactobacillus* bacterium carrying a cancer drug, for use in treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a
25 surface of the bacterium.

In a third aspect, disclosed herein is the use of an engineered *Lactobacillus* bacterium carrying a cancer drug in the manufacture of a medicament for treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.

30 In a fourth aspect, disclosed herein is a method of delivering a drug to a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying the drug, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.

In a fifth aspect, disclosed herein is a method of detecting a cancer in a subject, the method comprising administering an effective amount of an engineered *Lactobacillus* bacterium carrying a detectable label to the subject, wherein the detectable label is bound to a capture domain that is displayed on a surface of
35 the bacterium.

In a sixth aspect, disclosed herein is a method of labelling a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying a detectable label, wherein the detectable label is bound to a capture domain that is displayed on a surface of the bacterium.

5 In a seventh aspect, disclosed herein is an engineered *Lactobacillus* bacterium, comprising: a) a heterologous nucleic acid molecule encoding a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and b) a heterologous moiety that is bound to the capture domain. In some embodiments, the bacterium is *Lactobacillus plantarum* (Lp). In some embodiments, the Lp is of the strain WCFS1. 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
10 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 attached to the cell wall or cell membrane of the bacteria. The bacteria may contain one or more OppA polypeptides. In some embodiments, the OppA polypeptide
15 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.

In some embodiments, the heterologous moiety comprises a tag that is capable of binding to the capture domain. In some embodiments, the capture domain comprises a biotin-binding polypeptide and the tag is biotin. In some embodiments, the biotin-binding polypeptide is streptavidin. In some embodiments, the
20 heterologous moiety is a drug. In some embodiments, the drug is a prodrug comprising a cleavable entity. In some embodiments, the heterologous moiety comprises a detectable label. In some embodiments, the bacterium further comprises an OppA polypeptide on a surface of the bacterium.

In an eighth aspect, disclosed herein is a composition comprising a *Lactobacillus* bacterium of the seventh aspect. In some embodiments, the composition comprises one or more *Lactobacillus* bacteria.

25 In a ninth aspect, disclosed herein is a *Lactobacillus* bacterium of the seventh aspect or a composition as of the eighth aspect, for use as a medicament.

In a tenth aspect, disclosed herein is a method of preparing an engineered *Lactobacillus* bacterium of the seventh aspect, the method comprising: a) expressing in the bacterium a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and b) contacting the bacterium with a
30 heterologous moiety that it is capable of binding to the capture domain.

In an eleventh aspect, disclosed herein is a kit comprising the bacterium of the seventh aspect. In some embodiments, the kit comprises a first container containing a first composition according to the eighth aspect and a second container containing a second composition according to the eighth aspect, wherein the drug carried by the bacterium of the first composition comprises a different drug to the drug carried by
35 the bacterium of the second composition.

In one embodiment, the *Lactobacillus* bacteria is also engineered to produce and/or carry a drug for intranasal drug delivery to the olfactory region, brain and CNS. In preferred embodiments, the drug is a prodrug. In some embodiments, the bacteria are engineered to secrete the drug, preferably at an intranasal site (e.g., the olfactory epithelium) following intranasal administration. The bacteria may
5 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
10 accordance with the present methods is preferably expressed constitutively in the bacterium. The use of a constitutive 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 bacteria employed, i.e., one that is found natively in those bacteria. Preferably, the promoter directs expression at a level at
15 which the bacterial host cell remains viable, i.e., retains some metabolic activity, even if growth is not maintained.

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

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Summary of the Figures

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

25 **Figure 1** shows the binding affinity of various probiotic strains toward NPC cells. a) Quantification of the cancer binding capacity of probiotic strains using CNE-1 as the binding target. b) Lp localised on i) CNE-1, ii) CNE-2, iii) C666-1, iv) HK-1, and v) RPMI2650 cell surfaces and absent on vi) HNC cells. Red (highlighted with white triangles): *Lactobacillus plantarum* WCFS1 (Lp) expresses mCherry. Blue: mammalian nucleus stained by Hoechst. Green: cell cytoskeleton, cytoplasm, or microcilia of mammalian
30 cells. Scale bar: 25 µm. c) Quantification of Lp-cell binding affinity. The y-axis indicates the fold change in the FITC fluorescence signal, standardised to that of the HNC group. d) IF staining showing interactions between Lp and i) T24 bladder, ii) A549 lung, and iii) AGS gastric cancer cells. *Lactobacillus plantarum* WCFS1 (Lp) expresses mCherry (highlighted with white triangles). e) Inhibition of Lp-NPC binding by heparin salts in the coculture of Lp and four NPC cell lines. n = 5 experimental replicates. f) Schematic
35 diagram showing possible competitive inhibition between heparan sulfate and heparin.

Figure 2 shows identification of the OppA cancer binding protein from *L. plantarum* cell surface. a) Flow cytometric analysis showing binding affinity of OppA proteins towards all NPC cell lines. b) Increased Lp-NPC binding by the additional of Lp_0018 into coculture of *L. plantarum* and NPC cell lines. c) Alpha-fold prediction model of Lp_0018 and the simulation of its heparin docking complex. d) Schematics showing Lp_0018 enhancing Lp-NPC binding, and Lp_0018 SBD reducing Lp-NPC binding. e) Binding of Lp_0018 to the surface of Lp. f) Reduction of Lp-NPC binding by the addition of Lp_0018 SBD.

Figure 3 shows surface display of streptavidin and loading of the prodrug on *L. plantarum* WCFS1 (Lp). a) Expression cassette for Sav surface display in Lp. b) Schematics of tetramer Sav surface display. c) Western blot images showing the surface display of the Lp_1568-Sav fusion protein and secreted Sav protein. d) Detection of Lp_1568-Sav fusion protein on Lp cell surface via i) flow cytometry analysis and ii) IF. e) Detection of multimeric Sav on Lp cell surface via flow cytometry analysis and IF. f) Schematics showing the synthesis route of TL-SN and the loading of TL-SN. g) Loading of TL-SN on Lp-Sav and EV strain.

Figure 4 shows release of the prodrug 1 (SN) from Lp-Sav. a) & b) Schematic showing the mechanism of TL-SN activation and SN release. c) & f) Activation of TL-SN and release of SN in TL-SN-loaded Lp-Sav over time. d) & g) Dynamics of TL-SN activation by spectrum scanning over time. e) & h) Concentration of TL-SN in cell pellet and SN in supernatant pre- and post-activation.

Figure 5 shows *in vitro* characterisation of prodrug loaded Lp-Sav in NPC cells. a) Accumulation of SN in C666-1 following TL-SN activation, 24 hours incubation time. Red: β -actin stained by phalloidin. Blue: nucleus stained by Hoechst. Green: SN (highlighted with white triangles). Scale bars, 25 μ m. b) Viability of NPC cells after 24-hour co-culture with unloaded Lp-Sav at OD600 ~ 0.4. c) & d) IC50 of various treatments in NPC cells.

Figure 6 shows treatment of the xenograft mouse NPC model. a) Schematic of NPC xenograft mouse model for i) the evaluation of treatment effectiveness and ii) the study of Lp biodistribution. b) Table of the treatment groups used in this study. c) Visualisation of bioluminescent Lp in mouse organs at 24, 48, and 72 hours post i.v. injection (tumour radiance outlined in white). d) Bacteria density of Lp-CB in various mouse organs, including liver, spleen, tumour, caecum, heart, kidney, lung and stomach, 24, 48, and 72 hours after injection. *P < 0.05; ** P < 0.01; ***P < 0.001. e) Bacteria density of Lp-CB in blood 1, 3 and 6 h after injection. n = 3 mice (p value 1 h vs 3 h = 0.0261, 1 h vs 6 h = 0.0172, 3 h vs 6 h = 0.00029). f) Bacteria density of Lp-CB and LrD in tumour 24 hours postinjection. (p value = 0.0417) g) Tumour-to-liver ratio of Lp-CB and LrD 24 hours postinjection. (p value = 0.0280).

Figure 7 shows treatment of xenograft mouse NPC model. a) Body weight change of the mice in different groups during treatment. b) Tumour progression of mice in various treatment groups. Arrow indicates injection. c) Endpoint tumour burden of mice in various treatment groups.

Figure 8 is a schematic showing the delivery of prodrugs by engineered Lp to cancer cells. a) Lp recognises cancer cells via heparan sulfate binding. b) & c) Specific binding of Lp to cancer cells. d) Loading of biotinylated prodrugs on Lp and release of drugs by Lp in proximity to cancer cells.

Figure 9 shows SEM images of Lp co-cultured with the a CNE-1 monolayer. Lp were found on the surface of CNE-1 cells.

Figure 10 shows surface display of streptavidin in Lp using various anchor domains. a) Promoter library in Lp characterised by gusA assay. b). Replicon 256-based plasmids for genetic manipulation in Lp. c) Comparison of expression strength in pTRK-892 and pHSSC256 plasmids. d) Expression cassette for surface display of streptavidin. e) & f) Flow cytometry analysis and IF staining showing successful display of streptavidin through fusion to the transmembrane protein Lp_1568. Scale bar, 25 µm.

Figure 11 shows multiple sequence alignment of seven heparin-binding OppA proteins. Highly conserved residues are represented as red upper-case letters; weakly conserved residues are represented as blue lower-case letters; other residues are represented in black.

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.

The inventors have discovered that bacteria native to tumour microbiomes can be used for targeted delivery of anti-cancer drugs to cancer cells. Without being bound by theory, a variety of cancer tissues, including nasopharyngeal, breast, ovary and colorectal carcinomas, host commensal microbes that form unique cancer microbiota. These microbes have evolved intrinsic cancer-homing properties, and their metabolites directly impact the carcinogenesis, progression, and treatment of host tumour cells. This disclosure provides a drug delivery platform that harnesses bacteria-cancer interactions native to tumour microbiomes to achieve targeted delivery of anti-cancer drugs to cancer cells.

Lactobacillus strains are naturally present in the microbiome of many tumour and surrounding healthy tissues, for example in healthy and carcinomatous nasopharyngeal tissue. The inventors have identified a commensal *Lactobacillus plantarum* (Lp) strain (*Lactobacillus plantarum* WCFS1) with surprising affinity for various cancer cells. Without being bound by theory, the surface of this Lp strain contains OppA adhesin proteins with strong affinity for heparan sulphate, which is abundantly expressed on many cancer cells. The presence of several of these adhesins on *L. plantarum* makes this species particularly advantageous for targeting and colonising cancerous tissue compared to other *Lactobacillus* or commensal bacteria. Heterologous expression of OppA adhesins or coating of *L. plantarum* with exogenous adhesins (e.g., recombinantly expressed and purified OppA proteins) can further enhance cancer cell binding. For instance, the inventors have found that incubating an Lp strain with an OppA protein significantly increases the binding capability of the strain to nasopharyngeal carcinoma (NPC) cells.

Lactobacillus are commensal strains that are generally regarded as safe (GRAS) by the US FDA for consumption by humans and many animals. Although other bacteria such as strains from the *Clostridium*, *Escherichia*, *Listeria*, and *Salmonella* genera have been used for drug delivery, these bacteria are opportunistic pathogens that could potentially cause infections. In contrast, *Lactobacillus plantarum* (Lp) is cleared from non-cancerous tissues at much faster rates than strains like *Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *E. coli*, thus lowering the risk of bacterial infections following administration. *Lactobacillus* is also naturally present in many mucosa and they can thus be administered mucosally as another means of targeted delivery to avoid the side effects of systemic administration of payload drugs.

- 10 The inventors engineered *L. plantarum* (Lp) for drug delivery by expressing a capture domain on the bacterial surface which is able to bind suitably tagged drugs. The drugs can range from small molecules to proteins (e.g., antibodies) depending on the cancer to be treated. Having a capture domain on the bacterial surface allows the drug to be easily loaded onto the bacterium, ensures that the drug remains accessible to a target cell (e.g., a cancer cell), and keeps the drug separate from the internal environment of the bacteria, thereby maintaining the activity of the drug. The bacteria deliver the drug directly to cancer or tumour sites, thus reducing drug accumulation in healthy tissues. Cancer-targeted drug delivery that leverages the ability of *L. plantarum* to attach to cancer cells can enhance the efficiency of chemotherapy and reduce the intensity of chemotherapy regimens.

- 20 To further localise drug release to target sites and reduce systemic drug toxicity, the inventors modified the drugs into prodrugs that have little pharmacological activity until they are converted to an active form at a target site (e.g., a tumour site), e.g., in response to extracellular cues at the target site. The combination of targeted delivery using the Lp bacteria and localised drug activation using the prodrug format improves treatment specificity at target sites, thus reducing systemic side effects and making chemotherapy more tolerable for patients.

- 25 The Lp-based bacterial drug delivery platform can reduce the intensity of chemotherapy regimens in the treatment of a wide range of cancers. Firstly, as *L. plantarum* (Lp) strains are natural inhabitants of the microbiota in various human tissues, they can be used to deliver chemotherapy to cancers in these tissues through mucosal administration to reduce systemic toxicity of conventional chemotherapy. Secondly, targeted drug delivery by Lp can increase the availability of chemotherapy drugs to cancer cells, reduce damage to healthy tissues and improve treatment outcomes. Thirdly, Lp displaying streptavidin or biotin-binding moieties on its surface allows surface loading of various biotinylated chemotherapy drugs or prodrugs tailored to the unique characteristics and cellular microenvironments of particular cancers. The combination of Lp-mediated targeted delivery and localised prodrug activation can enhance the efficiency of chemotherapy and permit the use of a less intensive chemotherapy regimen.
- 35 Furthermore, the Lp bacteria can be administered to various mucosa as adjuvant or neoadjuvant therapy to sensitise cancer tissues to the standard chemotherapy.

Accordingly, this disclosure provides engineered *Lactobacillus* bacteria comprising a surface displayed capture domain capable of binding a heterologous moiety such as a drug or a detectable label, and

methods of using the engineered bacteria for detecting or treating cancer. Also provided are methods of preparing the bacteria.

Methods of treating cancer

- 5 Disclosed herein is a method of treating a cancer in a subject, the method comprising administering an effective amount of an engineered *Lactobacillus* bacterium carrying a drug to the subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.

In some embodiments, the bacterium is derived from a cancer tissue or a tissue where the cancer can occur. The bacterium may be a component of the native microbiome of the tissue or cancer tissue. The
10 tissue may be a mucosal tissue, e.g., the lining of the respiratory, gastrointestinal or genitourinary tracts. Examples of tissues from which the bacterium may be derived include but are not limited to nasal, nasopharyngeal, laryngeal, throat, tracheal, bronchial, oral, esophageal, gastric, intestinal, colorectal, bladder, urethral, vaginal, ovarian or breast tissue.

In one embodiment, the *Lactobacillus* bacterium is *Lactobacillus plantarum* (Lp). In one embodiment, the
15 *Lactobacillus* bacterium is *Lactobacillus plantarum* WCFS1.

In some embodiments, the bacterium further comprises an adhesin on a surface of the bacterium that is capable of attaching to a cancer cell. The adhesin may attach to an extracellular molecule on a surface of the cancer cell. The adhesin may attach to a polypeptide or polysaccharide. In some embodiments, the
adhesin is capable of attaching to a polysaccharide. In one embodiment, the polysaccharide is heparan
20 sulphate. In one embodiment, the adhesin is an OppA polypeptide.

In some embodiments, the bacterium comprises an OppA polypeptide on a surface of the bacterium. The OppA polypeptide can be any OppA polypeptide.

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
25 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
30 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%,
35 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.

In some embodiments, the *Lactobacillus* bacterium expresses a substrate binding domain (SBD) on a surface of the bacterium. The SBD is capable of binding to heparan sulphate. This enables *Lactobacillus* localisation to cancer cells, e.g., NPCs. In some embodiments, the SBD forms part of a polypeptide that can be presented on a surface of the *Lactobacillus* bacterium. For example, the SBD may form part of a transmembrane protein on the *Lactobacillus* bacterium surface or may be anchored on the surface membrane. In some embodiments, the OppA polypeptide comprises an SBD. In some embodiments, the OppA polypeptide consists of an SBD.

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 73 through to 453 of SEQ ID NO: 1. In some embodiments, the SBD 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 defined from position 73 through to 453 of SEQ ID NO: 1

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 82 through to 467 of SEQ ID NO: 2. In some embodiments, the SBD 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 defined from position 82 through to 467 of SEQ ID NO: 2.

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 87 through to 474 of SEQ ID NO: 3. In some embodiments, the SBD 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 defined from position 87 through to 474 of SEQ ID NO: 3.

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 87 through to 474 of SEQ ID NO: 4. In some embodiments, the SBD 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 defined from position 87 through to 474 of SEQ ID NO: 4.

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 79 through to 468 of SEQ ID NO: 5. In some embodiments, the SBD 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 defined from position 79 through to 468 of SEQ ID NO: 5.

In some embodiments, the SBD comprises a sequence as defined from amino acid position 76 through to 467 of SEQ ID NO: 6. In some embodiments, the SBD 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 defined from position 76 through to 467 of SEQ ID NO: 6.

In some embodiments, the SBD comprises an amino acid sequence as defined from amino acid position 82 through to 463 of SEQ ID NO: 7. In some embodiments, the SBD 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 defined from position 82 through to 463 of SEQ ID NO: 7.

5 **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>MKKRIWISSIAIVALGLVL</u> TGCGSQSSQKTLNVTATQSIATADPNK ADDIASQAAIAQFMEGLYTTNQKGKVVPAIAKKVVKPTNNGKTYTFNL RHNAKWSNGKKVTAADFVYSVQRQVDPNTKSQEVGHVAEIKNATAI TAGKKAVKTLGVKALGKYKLQITLQQRVPYFNYRLATEIYPLNKQFTE KYGNKYGTSAQRTLSNGPYVLKSWNGTNDTWKYVKNPYYYDRKQV RIKNIKVQTVKNNSTAENLFQSNEVQVTQITGTQVSAAEQGSLKSQM KVTKLNQLYFMLWNQKRSALQNTDLRRAVSYALNRQSLVKNVLKDG SVAATSLVPAGNTKNPTTGKDFNTDTGNLYPYSPAKAKQYWQKAQA SLGKQKLTQLLTNDNDINKSVAEYIQAIEKNLSGVTNVKSVPLTN EISTLSKGD FDFATLSWSSDFQDPVDFLNKASITNSVNF GKFNNAKY EQLISTITADKQSTKARYQTMQQA KLVAQQQGVTP IYQTAAHLISN KVGGVHFTLLRDALYRYAYWKE EQKLISEEDL (SEQ ID NO: 1)
Lp_0092 [UniProtKB - F9USS1 (F9USS1_LACPL)]	<u>MSLSGTHKLILTIGSVVIIGGIGFTVNAH</u> SHPATTKQSQVINLSAGSEIM SLDPAKMTDSVSNSQVTQVDEGLYRLNSQSQPVNALATKTTVTNGG HTYIIQLRHDARWSNGNRVTAHDFVYSWRRTLDPKSKSEFTYVFTNI KNADAIAAGKKKPSTLGVKANGDYQLTITLSKPVAYFKKILAVSTFYVPV NQPAVNKYGKKYGTSAATTVYNGPFTLTGWTGTNNTWTLKKNPAYY DHKLVLKHQINYQVIKSNTTAYNLYQSNKLD SVTLNGEQSVQNKNNQ ELKTLAGGQISFIQYNHHDHVAANRELRTALSLAINRSQLTN KVLENG SLPAKSFGVRNMAKNPKTG TDFVNDATVDGTVNYNLNQARAAYHKA QRQLGKKS LTLTITCGDDDN SHQVAEFIQGQLTSHLPGLKVTIKAMPF TAMLGKVS KGAFQLNLTSWSMDFADPSQSLTILTGDSNSNMGHYQS KAYDQAMQAADGPDALNATKRYQDLVQAAKTALTDQAVTPLYEGRS HLLVKSHLKGVIYNNFSGAANYRTAYVKE EQKLISEEDL (SEQ ID NO: 2)
Lp_0200 [UniProtKB - F9UT07 (F9UT07_LACPL)]	<u>MKWKVMLTTATAAA</u> STGLLLAAC SQGQSKSDNAGNLRAGMAQKQV LNWSENGSELTTLDTSLVTD SIANMINNTMEGLYRIGNNNKITPGIAT HTVISKDKKTYFTLRKDAKWSNGDPVTAQDFVYSWRRTIDPKTASQ YAYLYSGIKNADQITNGKAKANSLGIKADGKYKLTVTLDKPMAYFKLL MGFAIFFPQNQHAVQKYGKDYGTATKMVYDGPFTMKGWKGTNTT WSLNRNPKYWDKKHVYLEKINDQVVKSTTTGFNLFQSNKLD MATLS

	GEQVKNEKNNPKLVLRKTSRLNYLEFNQKKVPALANAKLRQAMSLTI DRHQLVTDVLADGSAVPKGFVTTGLATDPTTGEDFATENAVSAATT QDTAKAKKLWAEGLKETGKKSLLTLTHDNIDQTKETA EYVQGGQLEK ELPGLKITDITLPFKNRLARETSGNFQLAISGWQADFADPISDLGILTS TNDYNFGKWKNADYDAAVKQAEQSTDPTVRWTALGKAEKIIGTDEG VAPLTQTTLAQMVNPKLKGLIYNTSGTNYNFKDAYMAKE EQKLISEEDL (SEQ ID NO: 3)
Lp_0201 [UniProtKB - F9UT08 (F9UT08_LACPL)]	<u>MQWKLFATVATLTATTGVLLVGCGQSSSSSSNAGNTTAKMAKTQVL</u> NWSENGQDLTTLDPSLATDVISGTMISNSQEGLYRLGKNSKVTPGIA TKTTVSKDKKTYTFTLRKNAKWSNGDQVTAQDFVYGWRRTVNPKTA SQYAYLYSGIKNADKIVSGKAKTSTLGIKADGKYKLTVKLEKPMQYFK LLMGFVFFPQNHAVEKYGKDYGTSSSKMVYNGPFKMTGWKGTN STWTLKRNPYWDKKHVYLTIKIDQVVKSTTTAFNLFQSNKLDMATL SGQQVKNEKNNNKLVRKSSRLNYLEFNQKKVKLLANKKIRQAMSLT LNRKQLVNDVLGDGSVTPKGFVTTGLATDPTTGKDFATENTVKS AVT QDTTAKKLWKQGLKETGKKSVTLLTHDDTDQMKALAEYVQGGQLE KELPGLKVQSVTPYKTRISREIAGNFQLVISAWQADFADPVSDLGIM TSTNDYNFGKWTNKTFD SAINTANSTTSTTKRWQALATAEKT MATD QGVAPLSQNVIAQMVNPKLKGLVYNTAGINYNFKSAYMAKE EQKLISEEDL (SEQ ID NO: 4)
Lp_0783 [UniProtKB - F9UM05 (F9UM05_LACPL)]	<u>MKKRSVLAVVMSLVLVTLA</u> ACGKHNSQSSGNGKYASSQVLNLSYP SSLDSDISNMSGYSGTGNIFESLYRLGKNGSITPGLAKSTKVSKDGK TYTFTIRNAKWSDGSKITAQDFVYSWKRTVTPATKSQYAYLFSGVKN ADEIVAGKKSPSTLGVKAQGEHTFIVTLDKPITYFKKLMTYPLFGPISE KAVKKWGNKYATKAQYMLYSGPFKLTGWTGTNNSWQFVKNNQYW DKKAVHLQKINYTVNESTTTTLNLFQEKKDLTQLASEQVKNMKSSS DYTTYPSITAFLVYNFQDSNATIKKALNNAKIRQAISLSINRKT LVKNV IGDASTVSKTFVPQDLVKDAKTGKDFADESTVKNSTSYNKALAQKLW KQGLKETGIKKLSIQLLASNDEPNKPISQYLKSALEKNLDGLTVNLSNI PSKVASSRAQSGDFDLYLSGWGADFNDPISHLQIMTNNSGYNYGKY NSSTYNALVNKAQNQDANDTSARWQDMINAEKTIMKDQGITPLYQT VYSYLQNPVKVGIHNTAGTQWNYKYAYIAKE EQKLISEEDL (SEQ ID NO: 5)
Lp_1261 [UniProtKB - F9UN51 (F9UN51_LACPL)]	<u>MNFKTAAKVTVVAGASVLFLA</u> ACGSSKSSSSSKQTANWTESAELPT MDISKSTDVVSSNALNNTNEGLYRLGKNGKVTPGIAKSTKVTNNGGKT YTFTLRKNAKWSNGDKVTAKDFVYGWQRTVNPKTASQYAYLYSGIK NADAIVNGKKPVSSLGIKADGDYKLTVTLDKPIAYFDKLMGFVNFFPQ NEKVVKKYGSKYGTSSKAMVYNGPYKMTGWTGTNLSWTLKKNNNDY

	WDKKAVKMDSIKYQVVKDPSTGLNLFNSNKLDFTTLSNEQVKNYKN DKNYLVRKTASTFYVEFNRKDSDKTLAKAFQNKKIRQALSLAINRKS VNNTLGNGSTVSKGYVSEDLAKNPTTGKDFTTDAAVPSAVDYNLTK AKKLWKEGLKEIGETKLNKLLSDDTDAGKKATEFIQGGWEKLPGLTV TNQNVPFKTRLQRSQNGNFDTVISAWNADFTDPISFLSLFTSDNSYN NGKYDSAAYDDAVKKAEGADANNKTARWADMVAAEKQLMNDQGV PIYQQASATLTRSNLKGIIYNTAGSNYNFKYMSVKE EQKLISEEDL (SEQ ID NO: 6)
Lp_3686 [UniProtKB - F9ULM7 (F9ULM7_LACPL)]	<u>MQAKYTYSIIGVVSALLLTGCLGAVSNSSKTTSTADQNRTLGITIPGEP</u> MTTDPPTSJETNGGAVITQTGEGYRKNANNKIVAGVVEKKVKPTENK TRYTFTIRKNARWQNGTKITSQDFVTALRRQANPTTKSQNLDAISYIK NFDVAVNKGKMAVNKLGVTAINKRTFSIKLSKVPYMYEFTSFYPLNT EAVQKYGSKLGSNSATTVANGAYVIKGWKGSNDSWYYVKNKYYWN AKNVKIKRIKVTVTKDDNTAQNLFNAGTVQVTNISGQYVKNNANNSQ MTVTKTGRNNYIYFNSKKSVTANENFRHALSLVINQKTLAKDVLQDG SQAATNIVPKNYASDPTTGEDFVKEVGNLSPTNIKQAKQYWQKAQK ELGKQNVTLNFLCDDTDTEKKLAEYVQGVAEKYLKGVTIKIVAVPHAT HVS R DFTGNFDLVTVGWGPDPDAQNFLDGMQSSNSINFTQWKDA KYDALMAKVSNTAKYTAAQRWYEEKQADRRMLKLDVAPIPTYQASSA FLVSKDVGGGLQWDEFSGTSSQLQYAYWKE EQKLISEEDL (SEQ ID NO: 7)

A capture domain herein can be an oligonucleotide, a peptide, a polypeptide, a small molecule, or any combination thereof, that binds specifically to a desired heterologous moiety to be carried on the surface of the bacterium, or to a binding partner on the heterologous moiety. In preferred embodiments, the capture domain is a polypeptide. The capture domain may be a protein or protein fragment. The capture domain may be native to the *Lactobacillus* bacterium or a heterologous entity.

The *Lactobacillus* bacterium is engineered to display the capture domain on its surface. For display of a polypeptide capture domain, a heterologous nucleic acid molecule encoding the polypeptide may be introduced into the bacterium. In some embodiments, a plurality of heterologous nucleic acid molecules encoding a plurality of polypeptides may be introduced into the bacterium. In some embodiments, these polypeptides may encode the same capture domain. In other embodiments, these polypeptides may encode different capture domains.

A drug or heterologous moiety may be bound to the capture domain directly or through a binding partner on the drug or heterologous moiety that is recognised by the capture domain. In some embodiments, the drug comprises a tag that is capable of binding to the capture domain on the bacterial surface. The tag may be capable of covalent or non-covalent binding to the capture domain. The tag may be an oligonucleotide, a peptide, a polypeptide, a small molecule, or any combination thereof.

In one embodiment, the capture domain is a biotin-binding polypeptide, and the tag is biotin. The biotin-binding polypeptide may be endogenous or non-endogenous to the *Lactobacillus* bacterium.

Non-limiting examples of biotin-binding polypeptides include avidin, streptavidin, neutravidin, and homologues and derivatives thereof. "Homologues" refer to proteins which are functionally and/or structurally equivalent. Suitable homologues may be identified through amino acid sequence-based or protein domain-based comparative homology analysis using methods and databases known in the art. "Derivatives" may be protein variants engineered with improved properties, e.g., improved binding to biotin, greater thermo- or pH-stability, or improved or lack of multimer formation.

In one embodiment, the biotin-binding polypeptide is streptavidin. In one embodiment, the streptavidin is a multimeric streptavidin. In one embodiment, the multimeric streptavidin is a tetrameric streptavidin.

In some embodiments, the capture domain comprises an anchor domain for anchoring to the bacterial surface. The anchor domain may attach to the bacterial cell wall or cell membrane. In preferred embodiments, the anchor domain is a domain from an endogenous *Lactobacillus* protein.

In one embodiment, the anchor domain is a lipoprotein anchor domain. The lipoprotein anchor domain may be an anchor domain from Ip_1452 protein (Uniprot accession Q88X05).

In one embodiment, the anchor domain is a LysM anchor domain. The LysM anchor domain may be an anchor domain from Ip_3014 protein (Uniprot accession F9USE1).

In one embodiment, the anchor domain is a bacterial transmembrane domain. The bacterial transmembrane domain may be a transmembrane domain from Ip_1568 protein (Uniprot accession F9UNU7). In one embodiment, the bacterial transmembrane domain comprises an amino acid sequence having at least 70% sequence identity to an amino acid sequence as set forth in SEQ ID NO: 8.

Lp_1568 anchoring domain

MKLFFKITINRDPNPKSHIPFRLNFFIVFLLFAALIAQLAYLQVDYGGKFRSTVNSANNTTATGNVQRGSIY
DSTGRVLVGKSHQAIQYTKGLSVASTKMYEVANKLGEYLTPTDTLTDRNLADYYLAGAANLKSVAKKV
KTSSDDDLVLYAREVAYAEKHIINLTSTQKNAAIYYKMSSAYSLSTVNIKSTGVTSTELAEIGEHSQSEMPG
VKVGTSWTRSYPNGTDLSSVLGTVTTEKQGLPSDSIKTLAEGYSRDDSVGQSLEKQYENVLRGTSQ
TEVKTQDGVQKEIKKYGGQKGDNVQLTINSKFQKKVQSIIMSEAKTVGSSNAYSPGAYAVVMNPSTGAIY
ALAGASRNLTGKVTENALGTINQSFVMGSSVVGATVMGALQDGVITPTNSTLTDTPIKLAGTATKSSWF
NKNGGTSLSLNASTAMEVSSNSYMMQLAMKEGNFSYASGKALTMSNSVFSKLRGYFNEFGLGVKTGIDL
PGEASGYQGSSAQKDIGKALDLSYGNIDAYTTIQLAQYIATMANGGQRIAPHIVSAITGTSNGSQGAVKT
NVKPRVLNTIDVPSSYFDVWHQGYWDVWHGSMTQRTGSALASLSPAVAAKSGTAETFHGTTSTETLSLV
TYAPYKNPKVVIIVAFPGITATSGDYNMVVAKQIYAA (SEQ ID NO: 8)

The drug may be a polypeptide, nucleic acid, lipid, glycan, small molecule or a radionuclide. In some embodiments the drug comprises a cytotoxic agent, non-limiting examples of which include radionuclides; chemotherapeutic agents; growth inhibitory agents; enzymes and fragments thereof such as nucleolytic

enzymes; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; anti-microtubule agents, platinum coordination complexes, alkylating agents, antibiotic agents, topoisomerase I inhibitors, topoisomerase II inhibitors, antimetabolites, hormones and hormonal analogues, signal transduction pathway inhibitors, non-receptor
5 tyrosine kinase angiogenesis inhibitors, immunotherapeutic agents, pro-apoptotic agents, inhibitors of LDH-A, inhibitors of fatty acid biosynthesis, cell cycle signalling inhibitors, HDAC inhibitors, proteasome inhibitors, and inhibitors of cancer metabolism.

In some embodiments, the drug is a prodrug comprising a cleavable entity. Where the prodrug comprises a tag for binding to the capture domain on the bacterial surface, the cleavable entity may be a cleavable
10 linker between the drug and the tag.

In some embodiments, the prodrug is activated in a vicinity of a cancer cell or tissue through cleavage of the cleavable entity. For example, the cleavable entity may be cleaved in response to an extracellular cue in a vicinity of a cancer cell or tissue. The extracellular cue may be a cancer-specific cue. Non-limiting
15 examples of extracellular cues include an extracellular pH, an extracellular redox condition, an extracellular oxygen level and an extracellular metabolite, nucleic acid, peptide or polypeptide in the vicinity of the cancer cell or tissue.

In one embodiment, the cleavable entity is a redox-sensitive entity. The redox-sensitive entity may be sensitive to an oxidising agent or condition, or to a reductive agent or condition, present in the vicinity of a cancer cell or tissue. In one embodiment, the redox-sensitive entity is sensitive to an oxidising agent or
20 condition in a vicinity of a cancer cell or tissue, such as a reactive oxygen species (e.g., hydroperoxide, superoxide, hydroxyl radical, or singlet oxygen).

In one embodiment, the cleavable entity is an enzyme-cleavable entity. The enzyme-cleavable entity may be an enzyme-cleavable linker. The enzyme-cleavable linker may be, for example, a peptide or peptidic linker. The enzyme-cleavable entity may be cleavable by an extracellular enzyme in a vicinity of a cancer
25 cell or tissue, such as a matrix metalloproteinase (MMP).

In some embodiments, the bacterium is administered mucosally, peri-tumourally or intra-tumourally. Administration may be via injection, spray, direct application (e.g., direct application to a mucosal surface or tumour tissue) or implant (e.g., an implanted device or scaffold).

In one embodiment, the bacterium is administered mucosally. Mucosa as used herein can be any mucosa
30 such as oral mucosa, buccal mucosa, nasal mucosa, pulmonary mucosa, rectal mucosa, urethral mucosa, bladder mucosa, vaginal mucosa and ocular mucosa. Mucosal administration as used herein encompasses delivery to one or more mucosa. Mucosal administration includes but is not limited to oral administration, intranasal administration and bladder instillation. Oral mucosal administration includes buccal, sublingual and gingival routes of delivery.

35 In one embodiment, the bacterium is administered peri-tumorally, i.e., directly contacting or in close proximity to the tumour. In one embodiment, the bacterium is administered intra-tumourally, i.e., directly into a tumour mass. A skilled person may determine an appropriate route of administration based on the

site of the cancer, the properties of the *Lactobacillus* bacterium (such as the ability of the bacterium to home or adhere to a particular tissue or mucosa), the drug to be delivered, etc.

In some embodiments, the cancer is a carcinoma. Carcinomas are malignancies originating from epithelial cells, e.g., from epithelial cells lining the inner surfaces of the body, and are often associated with mucosal layers where native microbiomes are prevalent. Examples of carcinomas include but are not limited to malignant melanoma, non-small cell lung cancer (e.g., squamous non-small cell lung cancer and non-squamous non-small cell lung cancer), small cell lung cancer, head and neck cancer (e.g., oral cancer, nasal cancer, nasopharyngeal cancer, oropharyngeal cancer, hypopharyngeal cancer, laryngeal cancer, salivary gland cancer and tongue cancer), renal cell cancer (e.g., clear cell renal cell cancer), breast cancer, ovarian cancer (e.g., serous ovarian cancer and ovarian clear cell adenocarcinoma), uterine cancer (e.g., cervical cancer and endometrial cancer), anal cancer (e.g., anal canal cancer), colorectal cancer (e.g., MSI-H and/or dMMR positive colorectal cancer), hepatocellular cancer, esophageal cancer, gastric cancer, esophagogastric junction cancer, pancreatic cancer, urothelial cancer (e.g., bladder cancer, upper urinary tract cancer, ureteral cancer, renal pelvis cancer and urethral cancer), prostate cancer, fallopian tube cancer, primary peritoneal cancer, malignant pleural mesothelioma, gallbladder cancer, bile duct cancer, biliary tract cancer, skin cancer (e.g., uveal melanoma and Merkel cell carcinoma), testicular cancer (germ cell tumor), vaginal cancer, vulvar cancer, penile cancer, small intestine cancer, endocrine system cancer, thyroid cancer, parathyroid cancer, adrenal carcinoma, ocular retinoblastoma, neuroendocrine tumor, squamous cell carcinoma and the like.

In some embodiments, the cancer is a cancer of a mucosal tissue. Non-limiting examples of mucosal cancers include head and neck cancer (e.g., oral cancer, nasal cancer, nasopharyngeal cancer, oropharyngeal cancer, hypopharyngeal cancer, laryngeal cancer, salivary gland cancer and tongue cancer), lung cancer (e.g., small cell lung cancer, non-small cell lung cancer), esophageal cancer, esophagogastric junction cancer, gastric cancer, colorectal cancer, pancreatic duct cancer, uterine cancer (e.g., cervical cancer and endometrial cancer), ovarian cancer, bladder cancer, vaginal cancer, vulvar cancer, and anal cancer.

In some embodiments, the cancer is a nasopharyngeal, oral, lung, bladder, gastric, colorectal, skin, breast or ovarian cancer. In one embodiment, the cancer is a nasopharyngeal cancer, e.g., nasopharyngeal carcinoma.

Disclosed herein is an engineered *Lactobacillus* bacterium carrying a cancer drug, for use in treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.

Also disclosed herein is the use of an engineered *Lactobacillus* bacterium carrying a cancer drug in the manufacture of a medicament for treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium

Disclosed herein is a method of delivering a drug to a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying a cancer drug, wherein the cancer drug is bound to a

capture domain that is displayed on a surface of the bacterium. The method may be an *in vitro*, *ex vivo* or *in vivo* method.

Disclosed herein is a method of detecting a cancer in a subject, the method comprising administering an effective amount of an engineered *Lactobacillus* bacterium carrying a detectable label to the subject,
5 wherein the detectable label is bound to a capture domain that is displayed on a surface of the bacterium.

Disclosed herein is a method of labelling a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying a detectable label, wherein the detectable label is bound to a capture domain that is displayed on a surface of the bacterium.

The detectable label may be a radiolabel or an optically detectable label, such as a fluorescent label.

10

Engineered *Lactobacillus* bacteria

Disclosed herein is an engineered *Lactobacillus* bacterium, comprising: (a) a heterologous nucleic acid molecule encoding a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and (b) a heterologous moiety that is bound to the capture domain.

15 In one embodiment, the bacterium is *Lactobacillus plantarum* (Lp).

In some embodiments, the heterologous moiety comprises a tag that is capable of binding to the capture domain.

In some embodiments, the capture domain comprises a biotin-binding polypeptide and the tag is biotin.

In one embodiment, the biotin-binding polypeptide is streptavidin. In one embodiment, the streptavidin is
20 a multimeric streptavidin. In one embodiment, the multimeric streptavidin is a tetrameric streptavidin.

In some embodiments, the heterologous moiety is a drug. In one embodiment, the drug is a prodrug comprising a cleavable entity.

In some embodiments, the heterologous moiety comprises a detectable label.

In some embodiments, the bacterium further comprises an OppA polypeptide on a surface of the
25 bacterium. The bacterium may comprise one or more OppA polypeptides on the surface. 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.

Disclosed herein is a composition comprising a *Lactobacillus* bacterium as defined herein.

Disclosed herein is a *Lactobacillus* bacterium or a composition as defined herein, for use as a
30 medicament.

Disclosed herein is a method of preparing an engineered *Lactobacillus* bacterium as defined herein, the method comprising: a) expressing in the bacterium a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and b) contacting the bacterium with a heterologous moiety that it is capable of binding to the capture domain.

The method may further comprise introducing into the bacterium a heterologous nucleic acid encoding the polypeptide. The nucleic acid encoding the polypeptide may be operably linked to a promoter, e.g., an inducible or constitutive promoter. The nucleic acid may be comprised in a vector. The bacterium may be cultured under conditions suitable for expressing the polypeptide.

- 5 In one embodiment, the *Lactobacillus* bacteria is also engineered to produce and/or carry a drug for intranasal drug delivery to the olfactory region, brain and CNS. In preferred embodiments, the drug is a prodrug. In some embodiments, the bacteria are engineered to secrete the drug, preferably at an intranasal site (e.g., the olfactory epithelium) following intranasal administration. The bacteria may comprise one or more expression constructs encoding a heterologous drug molecule (e.g., a recombinant
- 10 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
- 15 constitutive 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 bacteria employed, i.e., one that is found natively in that bacteria. 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
- 20 maintained.

Definitions

- 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
- 25 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.

- As used herein, the term "subject" refers to a human or any non-human animal (e.g., mouse, rat, rabbit,
- 30 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.

- 35 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 is not natively encoded and/or 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.

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 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 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 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 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
5 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.

10 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
15 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
20 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
25 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
30 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.

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
35 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%.

Examples

5 EXAMPLE 1 - Prodrug-Conjugated Tumour-Seeking Commensals for Targeted Cancer Therapy

To identify a microbe that possesses cancer-binding capabilities, six commensal strains were investigated, including *Lactobacillus plantarum* WCFS1 and *Lactobacillus casei* DSM 20011, *Lactobacillus acidophilus* DSM 20079, *Lactobacillus reuteri* DSM 20016, *Lactobacillus salivarius* DSM 20555, and an *Escherichia coli* Nissle 1917 strain. The strains were selected based on their prevalence
10 within their higher species abundance in the nasopharynx, as well as their applications in otolaryngology. The binding affinity of these strains toward NPC were tested using the human NPC cell line CNE-1 as the target. As shown in Figure 1a, *L. plantarum* WCFS1 (Lp) exhibited the highest binding affinity toward CNE-1 cells compared to the other bacteria and showed a positive correlation with the confluence of the CNE-1 cells.

15 The binding efficacy of Lp to cancer cells and noncancerous cells of human nasal origin was then tested to further evaluate its specificity toward NPC. To visualise the binding, Lp was engineered to express a red fluorescent protein and the engineered strain was co-cultured with four NPC cell lines, including CNE-1, CNE-2, C666-1 and HK-1, a nasal squamous cell carcinoma line RPMI 2650, and a human healthy nasal cell line (HNC). Immunofluorescence staining (IF) of the Lp-NPC co-culture showed that Lp strain
20 bound to all cancer cells but not HNC cells (Fig. 1b). Through scanning electron microscopy (SEM), it was found that Lp was present on the surface of CNE-1 cells in co-culture and was not internalised by the host cells (Figure 9). In addition, Lp was labelled with fluorescein isothiocyanate (FITC) and the bacterial binding efficacy toward different cells was quantitatively compared. The binding efficacy of Lp to various cancer lines was 6 to 20 times higher than that to HNCs (Fig. 1c). Lp was also found to interact with
25 bladder, lung, and gastric cancer cells (Fig. 1d).

NPCs are known to overexpress heparan sulphate proteoglycans and have exposed heparan sulphate on the cell surface, which has been reported as a binding target for *Lactobacillus* adhesins. The respiratory epithelium in the nasal cavity does not present apical heparan sulfate, and ciliated HNCs that mimic the nasal epithelium display negligible binding. For these reasons, we hypothesised that heparan sulphate
30 serves as one of the binding targets in the Lp-NPC interaction.

For these reasons, it was hypothesized that heparan sulfate serves as one of the binding targets in the Lp-NPC interaction. To validate this hypothesis, we incubated cancer cells with a heparan sulfate analogue, heparin, prior to coculture. The addition of sodium heparin salt (HS) significantly inhibited Lp-NPC binding (Fig. 1e), which may be explained by the competitive inhibition of HS to the binding between
35 Lp and heparan sulfate (Fig. 1f). At the highest concentration, HS reduced the binding of Lp to all four NPC cell lines by 35–60% (Fig. 1e). Taken together, these data suggest that Lp can bind to NPCs specifically with high affinity, and heparan sulfate on NPCs may be the potential binding target for Lp.

Following that, we discovered a family of heparan sulphate proteins called oligopeptide binding proteins (OppA) in Lp that exhibit a high binding affinity to all NPCs. Seven homologous copies of OppA proteins were identified within the Lp genome. To investigate their potential for cancer cell targeting, we recombinantly expressed and purified all seven OppA proteins, namely Lp_0018, Lp_0092, Lp_0200, Lp_0201, Lp_0783, Lp_1261, and Lp_3686. To facilitate detection, we fused each OppA protein with a cMyc tag at the c-terminus. Flow cytometry analysis was conducted to determine the binding affinity of each OppA protein to five different types of cancer cells. The results showed that all OppA proteins bound to all five cancer cells, with Lp_0018 exhibiting the highest binding affinity and was a universal binder for all cancer cells, and Lp_0092 showing the lowest binding affinity (Fig. 2a). Notably, the OppA proteins displayed varying binding affinity to different cancer cell lines, suggesting that the presence of all seven proteins in Lp provides the bacteria with an overall higher binding affinity to a range of cancer cells.

We then examined whether such enhancement in binding is universal and could be observed in other cell lines. When added to the coculture, we found that Lp_0018 increased the bound bacteria in all NPC cells, and the increase was positively correlated with the concentration of Lp_0018 (Fig. 2b). At a concentration of 10 µg/ml, Lp_0018 increased the bound bacteria on various NPC cells by 8- to 27-fold (Fig. 2b). This finding was unexpected and contradicts a previous study on OppA proteins in *L. salivarius*. Interestingly, the addition of heparin salt in the coculture diminished the binding enhancement by Lp_0018 (Fig. 2b), which suggested that heparin blocked the binding of Lp_0018 to heparan sulfate on cancer cells. To investigate the interaction between OppA and heparan sulfate, we predicted the three-dimensional structure of all OppA proteins through AlphaFold42 and performed heparin docking on the simulated protein models using Cluspro43 with tetramer heparin as the ligand. Based on the docked complex analysis, all proteins are predicted to have potential heparin-binding capability and display two common heparin binding sites. Among the proteins, Lp_0783, Lp_0201 and Lp_1261 were predicted to have only one of the heparin binding sites, while Lp_0018, Lp_0092, Lp_0200 and Lp_3686 were shown to have both heparin binding sites. Among the OppA proteins, Lp_0018 was predicted to have much lower heparin binding energy at both sites, indicating a stronger interaction with the ligand heparin (Fig. 2c). From the superimposed docked complexes and sequence alignment, it was clear that contact map regions were highly conserved among the different OppA proteins. However, Lp_0018, in comparison to other OppA proteins, has many more arginine, lysine and asparagine residues, which are known to have strong interactions with heparin and heparan sulfate, in both binding sites (Fig. 2c), which accounts for a stronger heparin binding efficacy.

Both Lp_0200 and Lp_0018 themselves exhibited high binding efficacy towards CNE-1 cells (Figure 2a (i)). It was also observed that when incubated with Lp alone, both proteins adhered to the surface of the bacteria, although Lp_0018 exhibited much stronger adherence toward Lp than Lp_0200. This prompted us to hypothesize that the OppA proteins enhance Lp-NPC binding by adhering to Lp and NPCs simultaneously, and the weaker bacteria binding capacity of Lp_0200, likely due to the loss of the N-terminal sequences, limited its capacity to enhance Lp-CNE-1 binding (Fig. 2d). To verify this, we removed the N-terminal sequences of Lp_0018 and recombinantly expressed its substrate binding domain --Lp_0018 SBD. The loss of N-terminal sequences in Lp_0018 SBD demolished its binding

capability to Lp (Fig. 2e), and incubation of Lp_0018 SBD did not increase Lp-NPC binding in the coculture of any cell lines (Fig. 2f). This proves that recombinantly expressed Lp_0018

binds simultaneously to the host bacteria and NPCs. In addition, in four of the cell lines, the addition of Lp_0018 SBD reduced Lp-NPC binding, varying from a 25% reduction in RPMI2650 cells to a 65% reduction in C666-1 cells (Fig. 2f). The absence of reduction in CNE-1 cells suggests additional mechanisms in Lp-CNE-1 binding other than Lp_0018.

Accordingly, we conclude that OppA proteins are a major effector in the binding between Lp and NPCs, and heparan sulfate is the binding target on NPCs for OppA proteins.

Leveraging the cancer-binding characteristic of Lp, Lp was genetically modified to serve as a carrier strain for the delivery of prodrugs to cancer cells. Streptavidin (Sav) was chosen as the carrier protein for prodrugs due to the high binding affinity and specificity between tetramer streptavidin and biotinylated molecules. Lp was engineered to display a homo tetramer streptavidin (Lp-Sav) by combining a streptavidin surface displaying cassette (Lp_1568) with a streptavidin secretion cassette (as illustrated in Fig. 3a and b). Lp_1568-Sav was detected in both the control strain Lp-mSav and the Lp-Sav strains using cMyc tag detection by western blotting, flow cytometry analysis, IF staining targeting (as demonstrated in Fig. 3c,-3e). On the other hand, only the Lp-Sav strain displayed HA-tagged streptavidin on its surface, possibly as multimer streptavidin, as demonstrated in Figure 3e. This was further confirmed by western blot analyses, in which excessive Sav proteins were detected only in the culture supernatant of the Lp-Sav strain, which could interact with Lp_1568-Sav and multimerize into the tetramer streptavidin (Fig. 3c). Altogether, Lp was successfully engineered to exhibit active tetramer streptavidin protein on its bacterial surface, allowing for the surface loading of biotinylated molecules in the engineered strain.

A biotinylated prodrug TL-SN was developed, based on the chemotherapy agent SN-38 (referred to as SN), incorporating a thioketal linker that is sensitive to reactive oxygen species (ROS) and glutathione (GSH) (fig. 3f). When excited at 363nm, this prodrug generates distinctive emission peaks at 445nm, which helps to differentiation the prodrug TL-SN forms from native SN form. This prodrug showed a high binding capacity to the Lp-Sav strain, with the highest concentration achieved at 8.9 μ M (Fig. 3g).

The thioketal linker of TL-SN is sensitive to reactive oxygen species (ROS) and glutathione (GSH) (Fig. 4a, b). TL-SN was incubated with activators H₂O₂ or GSH to test the discharge of SN from Lp-Sav.

Interestingly, compared to GSH, TL-SN was more sensitive to H₂O₂ and released SN at a higher reaction rate in H₂O₂ (Fig. 4c, 4f). When excited at 363nm, the TL-SN prodrug generates distinctive emission peaks at 445nm, which helps differentiate the prodrug from native SN with an emission peak at 560nm. Using fluorescent signal measurements, it was observed that byproducts of TL-SN decomposition generated an emission peak at 420 nm, which contributed to the background fluorescent reading of TL-SN at 445 nm (Fig. 4d, 4g). Nevertheless, we measured the concentration of TL-SN and SN according to their fluorescent signal and found over 80% of the prodrug was converted to SN after 5 or 10 hours of incubation with H₂O₂ or GSH, indicating successful release of SN (Fig. 4e, 4h).

Prodrug-loaded Lp-Sav strain was co-cultured with C666-1 cells in the absence of ROS or GSH stimulants. The results showed that TL-SN-loaded Lp-Sav released SN in response to intrinsic activators from the C666-1 cells, resulting in the accumulation of SN within the C666-1 cells (Fig. 5a).

5 The anti-cancer efficacy of the prodrug-loaded Lp-Sav strains was evaluated *in vitro* by co-culturing them with four NPC cells and measuring the 24-hour half-maximal inhibitory concentration (IC₅₀). To inhibit bacterial growth during the co-culture, bacteriostatic antibiotics erythromycin and chloramphenicol were added to the media. Notably, the 24-hour co-culture did not impact the viability of the NPC cells (Fig. 5b). It was found that TL-SN had IC₅₀ values similar to SN in treating most NPC cells, with some cell lines showing higher IC₅₀ values for the prodrug (Fig. 5c & d). In some cell lines, the IC₅₀ values of prodrugs
10 were higher than SN (Fig. 5c & d), which is possibly owing to the delayed release of SN in prodrugs. Interestingly, TL-SN-loaded Lp-Sav cells resulted in a 5–10 fold reduction in IC₅₀ compared to SN in treating CNE-1, HK-1, and C666-1 cells. This reduction could be attributed to the potentially higher concentration of released SN around cancer cells. The

limited reduction observed in CNE-2 may be attributed to the higher resistance of CNE-2 cells to SN. TL-SN exhibited IC₅₀ values similar to SN in the treatment of most NPC cells and nearly 2 times the IC₅₀ of
15 SN in treating CNE-2 cells but the introduction of Lp-Sav improved treatment efficiency of TL-SN in all NPC cells, indicating a synergistic effect between Lp-Sav and TL-SN (Fig. 5c & 5d). Therefore, these results suggest that Lp could deliver TL-SN to NPC cells, leading to the accumulation of SN near the cells and improved treatment outcomes.

20 To further evaluate the performance of Lp-Sav in animal models, a xenograft NPC mouse model was established in BALB/C nude mice through subcutaneous injection of C666-1 cells (Fig 6a). Following the development of the xenograft tumour, Lp cells were administered through intravenous injection to study the biodistribution of Lp in tumour-bearing mice. Lp cells were engineered to express a bioluminescence protein and were injected through the tail vein to the tumour-bearing mice with considerably large
25 tumours. To study the biodistribution, the mice were euthanised 24, 48, and 72 hours post-injection and the organs were harvested for analysis. Through IVIS analysis, it was found that tumours generated the highest bioluminescence signal among all organs, which indicated a high amount of Lp localised in tumours (Fig. 6c). To quantify the amount of Lp in various organs, the organs were homogenised and their bacterial colony forming unit (CFU) in them was measured. It was found that liver, spleen, and
30 tumour had the highest amount of bacteria colonisation for Lp (Fig. 6d). In addition, the amount of Lp in tumours stably increased over 72 hours, while the bacteria cells in the liver, spleen, and other organs were mostly cleared in 48 hours (Fig. 6b). This clearance of Lp in non-tumoural organs occurs at a rate much faster than that of other tumour-seeking bacteria used in BMCT, including *Salmonella typhimurium* strains, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *E. coli*. Therefore, in comparison to many
35 opportunistic pathogens that are non-native to the human microbiome, Lactobacillus strains like Lp are readily available in the tumor microenvironment and serves as a safer choice for BMCT applications.

The transient presence of Lp-CB in the organs implies circulation of the bacteria in the mouse bloodstream. To assess the risk of sepsis, we determined the duration of Lp-CB presence in the blood by

monitoring the colony-forming units (CFU) of Lp-CB at 1, 3, and 6 h following intravenous injection (Fig. 6e). Notably, the level of Lp-CB rapidly declined in the mouse blood and became undetectable six hours post-injection (Fig. 6e).

To rule out the nonspecific localisation of bacteria in the tumour, the biodistribution of a control
5 *Lactobacillus* strain, *L. reuteri* DSM 20016 (referred to as LrD), in tumour-bearing mice was also evaluated. Following intravenous injection, 24 hours post-injection, the density of Lp-CB in tumour (Fig. 6f) and tumour-to-liver ratio of Lp-CB (Fig. 6fg) was over 35 times and 39 times higher than that of LrD, respectively, indicating higher specificity and efficiency in colonisation of NPC tumours.

Subsequently, the efficacy of Lp-Sav-TL-SN in treating NPCs in a xenograft mouse model was further
10 evaluated. It was hypothesised that with the present strategy, a lower dosage of the prodrug SN would be required to achieve significant inhibition of tumour growth. Therefore, a de-escalating treatment regimen was designed for the animal experiment: the treatments were administered at low doses at 50 µg/kg SN, given twice per week for two weeks, totaling 200 µg/kg SN (Fig. 6a). In comparison, previous studies reported the use of

15 Irinotecan, an FDA approved SN prodrug, at higher total doses ranging from 150 mg/kg to 400 mg/kg in less than four weeks.

To evaluate the efficacy of Lp-Sav-TL-SN, following the initial development of subcutaneous C666-1
tumours (5-8 mm in size), one treatment group (Lp-Sav-TL-SN) along with four control groups (PBS, Lp-Sav, SN, and TL-SN) were administered to the mice bearing the tumours via intravenous injection,
20 respectively (Fig. 6a and 6b). For each group, a total of four doses were administered to the mice over a span of two weeks, and no significant weight reduction or other side effects were observed in the mice (Fig. 7a). Based on the tumour volume and tumour weight-to-body weight ratio, the tumours in the mice in the Lp-Sav-TL-SN group were 50% smaller than those in the SN group, 66% smaller than those in the TL-SN group, and 75% smaller than those in the PBS control group (Fig. 7b and 7c).

25 In summary, the Lp-Sav-TL-SN strain significantly inhibited the progression of NPC tumours. The NPC recognition of native OppA proteins on the bacterial surface enabled bacteria to bind to the NPC surface, and the loaded TL-SN could release SN near the NPC cells to inhibit cancer growth. *In vitro* experiments showed that the prodrug-loaded microbes had significantly increased potency of SN against NPC cell lines, up to 10-fold higher. In a NPC mouse xenograft model, the delivery of prodrugs through engineered
30 Lp led to a 75% inhibition in tumour growth, significantly augmenting the efficacy of SN by 57%.

The present invention shows a reduction in potency in unloaded prodrug TLSN which exhibited a higher IC50 value against NPC cells *in vitro*. Treatment with TL-SN in tumor-bearing mice did not result in a significant reduction in tumor size. In contrast, the administration of Lp-Sav-TL-SN led to a drastic decrease in the IC50 and a significant reduction in tumor size compared to TL-SN and SN alone, marking
35 an immediate improvement in treatment potency. Additionally, the amount of SN needed in Lp-Sav-TL-SN to achieve significant tumor inhibition is 750 to 2,000 times lower than that in previous studies (Guichard et al, 1998; Kawato et al., 1991; Slatter et al., 2000; Godavarthi et al., 2023). The strategy of the present

invention could be an effective approach for treating cancer with reduced chemotherapy requirements and fewer associated side effects, making it a promising approach to de-escalate chemotherapy regimens.

By reprogramming Lp to display tetramer streptavidin proteins on the bacterial surface, the engineered strain Lp-Sav was generated. This modification allows for the surface loading of biotinylated prodrugs, offering a versatile platform for designing prodrugs with flexibility in their composition. This adaptability enables the selection of active drugs and release mechanisms tailored to the unique characteristics and sensitivities of individual cancers. Firstly, the surface loading pattern serves to protect the prodrugs from being metabolized by the intricate intracellular biochemistry within the bacteria. This protection expands the scope of potential chemotherapy drugs that can be employed. Secondly, the significant differences between bacterial and cancer metabolites make it possible to design linkers that respond to various cues within the tumor microenvironment, including hypoxia, acidosis, and high oxidative stress.

As described herein, the combination of Lp-Sav-TL-SN constitutes a comprehensive therapy that achieves both site-targeted delivery through Lp-Sav and site specific conversion of TL-SN through the embedded thioketal linker. This integrated system simplifies the design of prodrugs and broadens the range of candidate active drugs, making it a promising and versatile approach in cancer therapy.

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.

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.

Fu, Z., Li, S., Han, S., Shi, C. & Zhang, Y. Antibody drug conjugate: the “biological missile” for targeted cancer therapy. *Signal Transduct. Target. Ther.* 7, 93 (2022).

Xu, S. Internalization, trafficking, intracellular processing and actions of antibody-drug conjugates. *Pharm. Res.* 32, 3577–3583 (2015).

Khera, E. & Thurber, G. M. Pharmacokinetic and immunological considerations for expanding the therapeutic window of nextgeneration antibody–drug conjugates. *BioDrugs* 32,465–480 (2018)

Mecklenburg, L. A brief introduction to antibody–drug conjugates for toxicologic pathologists. *Toxicol. Pathol.* 46, 746–752 (2018).

- Cheng, Y. et al. Nanomaterials-induced toxicity on cardiac myocytes and tissues, and emerging toxicity assessment techniques. *Sci. Total Environ.* 800, 149584 (2021).
- 5 Tang, L.-L. et al. Global trends in incidence and mortality of nasopharyngeal carcinoma. *Cancer Lett* 374, 22–30 (2016).
- Zhang, Y., Rumgay, H., Li, M., Cao, S. & Chen, W. Nasopharyngeal Cancer Incidence and Mortality in 185 Countries in 2020 and the Projected Burden in 2040: Population-Based Global Epidemiological
10 Profiling. *JMIR Public Health Surveill* 9, e49968 (2023).
- McDowell, L., Corry, J., Ringash, J. & Rischin, D. Quality of life, toxicity and unmet needs in nasopharyngeal cancer survivors. *Front. Oncol.* 10 (2020).
- 15 Wang, C. et al. Toxicities of chemoradiotherapy and radiotherapy in nasopharyngeal carcinoma: an updated meta-analysis. *J. Int. Med. Res.* 47, 2832–2847 (2019).
- Guichard, S. et al. Comparison of the pharmacokinetics and efficacy of irinotecan after administration by the intravenous versus intraperitoneal route in mice. *Cancer Chemother. Pharmacol.* 42, 165–170 (1998).
20 Kawato, Y. et al. Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother. Pharmacol* 28, 192–198 (1991).
- Slatter, J. G. et al. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V.
25 infusion of [(14)C]CPT-11 in cancer patients. *Drug Metab. Dispos* 28, 423–433 (2000).
- Godavarthi, J. D. et al. The synergistic antitumor effect of irinotecan and flavonoids on human colon cancer xenograft mice. *J. Pharmacol. Exp. Ther.* 385, 44 (2023).
- 30 For standard molecular biology techniques, see Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Claims:

1. A method of treating a cancer in a subject, the method comprising administering an effective amount of an engineered *Lactobacillus* bacterium carrying a drug to the subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.
5
2. The method of claim 1, wherein the bacterium is derived from a cancer tissue or a tissue where the cancer can occur.
3. The method of claim 1 or 2, wherein the bacterium is *Lactobacillus plantarum* (Lp).
10
4. The method of any one of claims 1 to 3, wherein the bacterium comprises an OppA polypeptide on a surface of the bacterium.
5. The method of claim 4, 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 NO: 1–7.
15
6. The method of any one of claims 1 to 5, wherein the drug comprises a tag that is capable of binding to the capture domain.
20
7. The method of claim 6, wherein the capture domain is a biotin-binding polypeptide, and the tag is biotin.
8. The method of claim 7, wherein the biotin-binding polypeptide is streptavidin.
25
9. The method of any one of claims 1 to 8, wherein the capture domain comprises an anchor domain for anchoring to the bacterial surface.
10. The method of claim 9, wherein the anchor domain is a bacterial transmembrane domain.
30
11. The method of claim 10, wherein the bacterial transmembrane domain 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: 8.
- 35 12. The method of any one of claims 1 to 11, wherein the drug is a prodrug comprising a cleavable entity.
13. The method of claim 12, wherein the prodrug is activated in a vicinity of a cancer cell or tissue through cleavage of the cleavable entity.
- 40 14. The method of claims 12 or 13, wherein the cleavable entity is a redox-sensitive entity.

15. The method of any one of claims 1 to 14, wherein the bacterium is administered mucosally, peritumourally or intra-tumourally.
16. The method of any one of claims 1 to 15, wherein the cancer is a carcinoma.
17. The method of any one of claims 1 to 16, wherein the cancer is a nasopharyngeal, oral, lung, bladder, gastric, colorectal, skin, breast or ovarian cancer.
18. The method of claim 17, where the cancer is a nasopharyngeal cancer.
19. An engineered *Lactobacillus* bacterium carrying a cancer drug, for use in treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.
20. Use of an engineered *Lactobacillus* bacterium carrying a cancer drug in the manufacture of a medicament for treating a cancer in a subject, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.
21. A method of delivering a drug to a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying the drug, wherein the drug is bound to a capture domain that is displayed on a surface of the bacterium.
22. A method of detecting a cancer in a subject, the method comprising administering an effective amount of an engineered *Lactobacillus* bacterium carrying a detectable label to the subject, wherein the detectable label is bound to a capture domain that is displayed on a surface of the bacterium.
23. A method of labelling a cancer cell, the method comprising contacting the cancer cell with a *Lactobacillus* bacterium carrying a detectable label, wherein the detectable label is bound to a capture domain that is displayed on a surface of the bacterium.
24. An engineered *Lactobacillus* bacterium, comprising:
- a) a heterologous nucleic acid molecule encoding a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and
 - b) a heterologous moiety that is bound to the capture domain.
25. The engineered bacterium of claim 24, wherein the bacterium is *Lactobacillus plantarum* (Lp).
26. The engineered bacterium of claims 24 or 25, wherein the heterologous moiety comprises a tag that is capable of binding to the capture domain.
27. The engineered bacterium of claim 26, wherein the capture domain comprises a biotin-binding polypeptide and the tag is biotin.

28. The engineered bacterium of claim 27, wherein the biotin-binding polypeptide is streptavidin.
29. The engineered bacterium of any one of claims 24 to 28, wherein the heterologous moiety is a drug.
- 5 30. The engineered bacterium of claim 29, wherein the drug is a prodrug comprising a cleavable entity.
31. The engineered bacterium of any one of claims 24 to 30, wherein the heterologous moiety comprises a detectable label.
- 10 32. The engineered bacterium of any one of claim 24 to 31, wherein the bacterium further comprises an OppA polypeptide on a surface of the bacterium.
33. A composition comprising a *Lactobacillus* bacterium of any one of claims 24 to 32.
- 15 34. The composition of claim 33, comprising one or more *Lactobacillus* bacteria.
35. A *Lactobacillus* bacterium of any one of claims 24 to 32, or a composition of claim 33, for use as a medicament.
- 20 36. A method of preparing an engineered *Lactobacillus* bacterium according to any one of claims 24 to 32, the method comprising:
- 25 a) expressing in the bacterium a polypeptide comprising a capture domain that is displayed on a surface of the bacterium; and
- b) contacting the bacterium with a heterologous moiety that it is capable of binding to the capture domain.
37. A kit comprising the bacterium of any one of claims 24 to 32.
- 30 38. The kit of claim 37, comprising a first container containing a first composition according to any one of claims 33 or 34 and a second container containing a second composition according to any one of claims 33 or 34, wherein the drug carried by the bacterium of the first composition comprises a different drug to the drug carried by the bacterium of the second composition.
- 35 39. The kit of claim 38, further comprising a third container containing a third composition according to any one of claims 33 or 34, 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.
- 40