

Compositions And Methods For Treating Diseases and Disorders Using *Harryflintia****Acetispora*****RELATED APPLICATIONS**

[1] This application claims the benefit of priority to U.S. Provisional Patent Applications having serial numbers 63/037,882, filed June 11, 2020, and 63/091,015, filed October 13, 2020, the contents of each are hereby incorporated by reference in their entirety.

SUMMARY

[2] Provided herein are methods and compositions related to the use of *Harryflintia acetispora* bacteria and/or microbial extracellular vesicles (mEVs) from *Harryflintia acetispora* bacteria in the treatment and/or prevention of a disease or a health disorder (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, and/or a metabolic disease). As disclosed herein, certain types of mEVs such as secreted microbial extracellular vesicles (smEVs) or processed microbial extracellular vesicles (pmEVs) obtained from *Harryflintia acetispora* bacteria have therapeutic effects and are useful for the treatment and/or prevention of a disease or a health disorder (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, and/or a metabolic disease).

[3] In certain aspects, provided herein are pharmaceutical compositions comprising *Harryflintia acetispora* bacteria, *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs), or any combination thereof.

[4] In some embodiments, a pharmaceutical composition provided herein comprises *Harryflintia acetispora* bacteria (e.g., live bacteria, killed bacteria, attenuated bacteria). In some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs). For example, in some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* smEVs. In some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* pmEVs. In some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* smEVs and *Harryflintia acetispora* pmEVs. In some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* bacteria and *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs). For example, in some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* bacteria and *Harryflintia acetispora* smEVs. In some embodiments, a pharmaceutical composition comprises *Harryflintia acetispora* bacteria and *Harryflintia acetispora* pmEVs. In some embodiments, a pharmaceutical composition

comprises *Harryflintia acetispora* bacteria, *Harryflintia acetispora* smEVs, and *Harryflintia acetispora* pmEVs.

[5] A pharmaceutical composition comprising mEVs can contain smEVs, pmEVs or a combination of both.

[6] In some embodiments, the *Harryflintia acetispora* strain is a strain comprising at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity (e.g., at least 99.5% sequence identity, at least 99.6% sequence identity, at least 99.7% sequence identity, at least 99.8% sequence identity, at least 99.9% sequence identity) to the nucleotide sequence (e.g., genomic sequence, 16S sequence, and/or CRISPR sequence) of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).

[7] In some embodiments, the *Harryflintia acetispora* strain is a strain comprising at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% 16S sequence identity (e.g., at least 99.5% sequence identity, at least 99.6% sequence identity, at least 99.7% sequence identity, at least 99.8% sequence identity, at least 99.9% sequence identity) to SEQ ID NO: 1.

[8] In some embodiments, the *Harryflintia acetispora* strain is the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).

[9] In some embodiments, a pharmaceutical composition comprises at least 1×10^6 , 1×10^7 , or 1×10^8 colony forming units (CFUs) of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A) whole bacteria.

[10] In some embodiments, a pharmaceutical composition comprises 1×10^6 to 1×10^{12} total cells (e.g., as determined by total cell count (TCC)) of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A) whole bacteria. In some embodiments, a pharmaceutical composition comprises at least 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} , 1×10^{11} , or 1×10^{12} total cells (e.g., as determined by total cell count (TCC)) of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A) whole bacteria.

[11] In some embodiments, the whole bacteria may be live, killed, attenuated, lyophilized, or irradiated (e.g., UV or gamma irradiated).

[12] In some embodiments, a pharmaceutical composition comprises secreted mEVs (smEVs).

[13] In some embodiments, a pharmaceutical composition comprises processed mEVs (pmEVs).

[14] In some embodiments, a pharmaceutical composition comprises pmEVs and the pmEVs are produced from live bacteria. In some embodiments, the pmEVs are produced from dead bacteria. In some embodiments, the pmEVs are produced from bacteria that have been gamma irradiated, UV irradiated, heat inactivated, acid treated, or oxygen sparged. In some embodiments, the pmEVs are produced from non-replicating bacteria.

[15] In some embodiments, a pharmaceutical composition comprises mEVs (such as smEVs and/or pmEVs) that are lyophilized (e.g., the lyophilized product further comprises a pharmaceutically acceptable excipient). In some embodiments, the mEVs (such as smEVs and/or pmEVs) are gamma irradiated. In some embodiments, the mEVs (such as smEVs and/or pmEVs) are UV irradiated. In some embodiments, the mEVs (such as smEVs and/or pmEVs) are heat inactivated (e.g., at 50°C for two hours or at 90°C for two hours). In some embodiments, the mEVs (such as smEVs and/or pmEVs) are acid treated. In some embodiments, the mEVs (such as smEVs and/or pmEVs) are oxygen sparged (e.g., at 0.1 vvm for two hours).

[16] In some embodiments, a pharmaceutical composition comprises a dose of mEVs (such as smEVs and/or pmEVs) of about 2×10^6 to about 2×10^{16} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)). In some embodiments, the dose of mEVs (such as smEVs and/or pmEVs) is about 1×10^7 - about 1×10^{15} particles, e.g., as measured by NTA. In some embodiments, a pharmaceutical composition comprises a dose of mEVs (such as smEVs and/or pmEVs) of about 5 mg to about 900 mg total protein (e.g., wherein total protein is determined by Bradford assay or BCA assay).

[17] In certain aspect, a pharmaceutical composition provided herein comprises *Harryflintia acetispora* microbial extracellular vesicles (mEVs) (such as smEVs and/or pmEVs) and *Harryflintia acetispora* bacteria.

[18] In some embodiments, the pharmaceutical composition comprises smEVs and the smEVs are produced from live bacteria. In some embodiments, the pharmaceutical composition comprises smEVs and the smEVs are produced from a high yield strain of *Harryflintia acetispora* bacteria. In some embodiments, the high yield strain produces at least 3×10^{13} mEVs per liter from a bioreactor-grown culture.

[19] In some embodiments, the pharmaceutical composition comprises smEVs and the smEVs are from one strain of *Harryflintia acetispora* bacteria.

[20] In some embodiments, the pharmaceutical composition comprises mEVs and the mEVs are from one strain of *Harryflintia acetispora* bacteria.

[21] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[22] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* bacteria particles.

[23] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total proteins in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[24] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%,

49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total proteins in the pharmaceutical composition are *Harryflintia acetispora* bacteria proteins.

[25] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[26] In some embodiments, a pharmaceutical composition comprises at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* bacteria lipids.

[27] In certain aspects, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be used for the treatment or prevention of a disease. In some embodiments, the disease is an immune disorder (e.g., a cancer, an autoimmune disease, an inflammatory disease, or a metabolic disease), an inflammatory disorder (e.g., dermatitis), a cancer (e.g., colorectal cancer), or a dysbiosis.

[28] In certain embodiments, provided herein are methods of treating a subject who has cancer comprising administering to the subject a pharmaceutical composition described herein.

[29] In certain embodiments, provided herein are methods of treating a subject who has dysbiosis comprising administering to the subject a pharmaceutical composition described herein. In certain embodiments, provided herein are methods of treating a subject who has an immune disorder (e.g., an autoimmune disease, an inflammatory disease, an allergy) comprising administering to the subject a pharmaceutical composition described herein. In certain embodiments, provided herein are methods of treating a subject who has a metabolic disease comprising administering to the subject a pharmaceutical composition described herein. In certain embodiments, provided herein are methods of treating a subject who has a neurologic disease comprising administering to the subject a pharmaceutical composition described herein. In some embodiments, the pharmaceutical composition described herein is administered once a day. In some embodiments, the pharmaceutical composition described herein is administered twice a day. In some embodiments, the pharmaceutical composition described herein is formulated for a daily dose. In some embodiments, the pharmaceutical composition described herein is formulated for twice a day dose, wherein each dose is half of the daily dose.

[30] In preferred embodiments, a pharmaceutical composition provided herein induces an immune response. In some embodiments, a pharmaceutical composition activates innate antigen presenting cells.

[31] In some embodiments, a pharmaceutical composition provided herein has one or more beneficial immune effects outside the gastrointestinal tract, e.g., when orally administered. In some embodiments, a pharmaceutical composition modulates immune effects outside the gastrointestinal tract in the subject, e.g., when orally administered.

[32] In certain aspects, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, is formulated for oral, rectal, sublingual, topical intradermal, intraperitoneal, or subcutaneous administration.

[33] In some embodiments, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be prepared as powder (e.g., for resuspension) or as a solid dose form, such as a tablet, a minitab, a capsule, a pill, or a powder; or a combination of these forms (e.g., minitabts comprised in a capsule). In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

[34] In some embodiments, a pharmaceutical composition provided herein can comprise lyophilized *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof. The lyophilized *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be formulated into a solid dose form (optionally comprising an enteric coating), such as a tablet, a minitab, a capsule, a pill, or a powder; or can be resuspended in a solution (optionally further comprising a pharmaceutical excipient (e.g., sucrose or glucose)).

[35] In some embodiments, a pharmaceutical composition provided herein can comprise gamma irradiated *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof. The gamma irradiated *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be formulated into a solid dose form (optionally comprising an enteric coating), such as a tablet, a minitab, a capsule, a pill, or a powder; or can be resuspended in a solution (optionally further comprising a pharmaceutical excipient (e.g., sucrose or glucose)).

[36] In some embodiments, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be orally administered. In some embodiments, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be topically administered. In some embodiments, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be administered intravenously. In some embodiments, a pharmaceutical composition provided herein comprising *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, can be administered intratumorally or subcutaneously, e.g., to a subject who has a tumor.

[37] In certain aspects, provided herein are methods of making and/or identifying *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, that are useful for treatment and/or prevention of a disease.

[38] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, are obtained from *Harryflintia acetispora* bacteria that have been selected based on certain desirable properties, such as reduced toxicity and adverse effects (e.g., by removing or deleting lipopolysaccharide (LPS)), enhanced oral delivery (e.g., by improving acid resistance, muco-adherence and/or penetration and/or resistance to bile

acids, resistance to anti-microbial peptides and/or antibody neutralization), target desired cell types (e.g., M-cells, goblet cells, enterocytes, dendritic cells, macrophages), improved bioavailability systemically or in an appropriate niche (e.g., mesenteric lymph nodes, Peyer's patches, lamina propria, tumor draining lymph nodes, and/or blood), enhanced immunomodulatory and/or therapeutic effect (e.g., either alone or in combination with another therapeutic agent), enhanced immune activation, and/or manufacturing attributes (e.g., growth characteristics, yield, greater stability, improved freeze-thaw tolerance, shorter generation times).

[39] In some embodiments, the mEVs (such as smEVs and/or pmEVs) are from engineered *Harryflintia acetispora* bacteria that are modified to enhance certain desirable properties. In some embodiments, the engineered *Harryflintia acetispora* bacteria are modified so that mEVs (such as smEVs and/or pmEVs), bacteria for pharmaceutical composition, or any combination thereof, produced therefrom will have reduced toxicity and adverse effects (e.g., by removing or deleting lipopolysaccharide (LPS)), enhanced oral delivery (e.g., by improving acid resistance, muco-adherence and/or penetration and/or resistance to bile acids, resistance to anti-microbial peptides and/or antibody neutralization), target desired cell types (e.g., M-cells, goblet cells, enterocytes, dendritic cells, macrophages), improved bioavailability systemically or in an appropriate niche (e.g., mesenteric lymph nodes, Peyer's patches, lamina propria, tumor draining lymph nodes, and/or blood), enhanced immunomodulatory and/or therapeutic effect (e.g., either alone or in combination with another therapeutic agent), enhanced immune activation, and/or improved manufacturing attributes (e.g., growth characteristics, yield, greater stability, improved freeze-thaw tolerance, shorter generation times). In some embodiments, provided herein are methods of making such mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof.

[40] In certain aspects, provided herein is use of a pharmaceutical composition described herein for the preparation of a medicament for treatment (or prevention) of a condition described herein, e.g., a cancer, an immune disorder, a metabolic disease, a neurologic disease, an inflammatory disease, or a dysbiosis, e.g., as described herein.

[41] In certain aspects, provided herein is a pharmaceutical composition described herein for use in treating (or preventing) of a condition described herein, e.g., a cancer, an immune disorder, a metabolic disease, a neurologic disease, an inflammatory disease, or a dysbiosis, e.g., as described herein.

[42] In certain aspects, provided herein is a method of using the pharmaceutical compositions described herein in treating a subject (e.g., human) in need thereof.

[43] In some embodiments, a method treats or prevents a disease in a subject, the method comprising administering to the subject at least one pharmaceutical composition described herein. In some embodiments, a use of at least one pharmaceutical composition described herein is for treating or preventing a disease in a subject. Non-limiting examples of the disease include an immune disorder (e.g., a cancer, an autoimmune disease, an inflammatory disease, or a metabolic disease), an inflammatory disorder (e.g., dermatitis), a cancer (e.g., colorectal cancer), or a dysbiosis. In some embodiments, the pharmaceutical composition described herein is administered once a day. In some embodiments, the pharmaceutical composition described herein is administered twice a day. In some embodiments, the pharmaceutical composition described herein is formulated for a daily dose. In some embodiments, the pharmaceutical composition described herein is formulated for twice a day dose, wherein each dose is half of the daily dose.

[44] In some embodiments, a method or use of a pharmaceutical composition provided herein further comprises administering to the subject an additional therapy. In some embodiments, the additional therapy is an antibiotic. In some embodiments, the method or use further comprises administering to the subject one or more other cancer therapies (e.g., surgical removal of a tumor; administration of a chemotherapeutic agent, radiation therapy, and/or a cancer immunotherapy including but not limited to an immune checkpoint inhibitor, a cancer-specific antibody, a cancer vaccine, a primed antigen presenting cell, a cancer-specific T cell, a cancer-specific chimeric antigen receptor (CAR) T cell, an immune activating protein, and/or an adjuvant). In some embodiments, the method or use further comprises the administration of another therapeutic bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, from one or more other bacterial strains (e.g., therapeutic bacteria). In some embodiments, the method further comprises the administration of an immune suppressant and/or an anti-inflammatory agent. In some embodiments, the method further comprises the administration of a metabolic disease therapeutic agent.

[45] In certain aspects, further provided herein is a method of preparing a pharmaceutical composition described herein in a suspension, the method comprising: combining *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, with a pharmaceutically acceptable buffer (e.g., PBS); thereby preparing the

pharmaceutical composition. In some embodiments, the suspension further comprises sucrose or glucose. In some embodiments, the *Harryflintia acetispora* mEVs, bacteria, or any combination thereof are comprised in a dry form (e.g., powder) that is combined with the pharmaceutically acceptable buffer. In some embodiments, the *Harryflintia acetispora* mEVs, bacteria, or any combination thereof are comprised in a wet form (e.g., biomass or pellet or other liquid) that is combined with the pharmaceutically acceptable buffer.

[46] In certain aspects, also provided herein is a method of preparing a pharmaceutical composition described herein in a solid dose form, the method comprising: (a) combining *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, with a pharmaceutically acceptable excipient, and (b) compressing the *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, and a pharmaceutically acceptable excipient, thereby preparing the pharmaceutical composition. In some embodiments, the method further comprises enterically coating the solid dose form. In some embodiments, the *Harryflintia acetispora* mEVs, bacteria, or any combination thereof are comprised in a dry form (e.g., powder) that is combined with the pharmaceutically acceptable excipient.

[47] A pharmaceutical composition provided herein can deliver a therapeutically effective amount of *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, to a subject (e.g., a human) in need thereof. Similarly, a pharmaceutical composition provided herein can deliver a non-natural amount of the therapeutically effective amount of *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof, to a subject (e.g., a human) in need thereof. Such pharmaceutical composition can bring benefits to a subject (e.g., a human), such as treating and/or preventing a disease or a healthy disorder.

[48] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof reduce tumor growth in a CT26 preclinical model of cancer.

[49] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof reduce ear thickness in a DTH (delayed type hypersensitivity) preclinical model of inflammation.

[50] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof reduce ear thickness in a FITC (fluorescein isothiocyanate) cutaneous hypersensitivity preclinical model of inflammation.

[51] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce cytokine production from PMA-differentiated U937 cells. In some embodiments, the *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or the pharmaceutical composition induce production of one or more of: IL-10; TNF- α ; IL-6; IP-10; and IL-1 β (e.g., as compared to a blank control).

[52] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof reduce cytokine production in mesenteric lymph nodes (mLNs), e.g., in a FITC (fluorescein isothiocyanate) cutaneous hypersensitivity preclinical model. In some embodiments, the *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or the pharmaceutical composition reduce production of one or more of: IL-4, IL-5, IL-13, IL-10, and IL-33 in the mesenteric lymph nodes (mLNs).

[53] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce engagement of the TLR2 receptor.

[54] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce IL-10R engagement.

[55] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce modulation of lymphocytes, e.g., to a more anti-inflammatory state in the gut lymphoid tissue.

[56] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce modulation of B cell lymphocytes, e.g., to modulate anti-inflammatory effects.

[57] In certain aspects, *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof and/or a pharmaceutical composition comprising the bacteria, mEVs (such as smEVs and/or pmEVs), or any combination thereof induce modulation of CD4+ T cells, e.g., to an anti-inflammatory state.

BRIEF DESCRIPTION OF THE FIGURES

[58] Fig. 1 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model at day 22. smEVs from *Harryflintia acetispora* Strain A cultured in glucose-containing media is compared with the smEVs from *Harryflintia acetispora* Strain A cultured in N-acetylglucosamine (NAG)-containing media. Welch's test is performed for treatment vs. vehicle.

[59] Fig. 2 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model over a period of time. smEVs from *Harryflintia acetispora* Strain A cultured in glucose-containing media is compared with the smEVs from *Harryflintia acetispora* Strain A cultured in N-acetylglucosamine (NAG)-containing media. Welch's test is performed for treatment vs. vehicle.

[60] Fig. 3 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model at day 23. Welch's test is performed for treatment vs. vehicle.

[61] Fig. 4 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model over a period of time. Welch's test is performed for treatment vs. vehicle.

[62] Fig. 5 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model at day 21. Welch's test is performed for treatment vs. vehicle.

[63] Fig. 6 shows the efficacy of *Harryflintia acetispora* Strain A smEVs as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model over a period of time. Welch's test is performed for treatment vs. vehicle.

[64] Fig. 7 shows the efficacy of *Harryflintia acetispora* Strain A whole bacteria as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model at day 24. Welch's test is performed for treatment vs. vehicle.

[65] Fig. 8 shows the efficacy of *Harryflintia acetispora* Strain A whole bacteria as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model over a period of time. Welch's test is performed for treatment vs. vehicle.

[66] Fig. 9 shows the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at two doses, $2E+11$ and $2E+07$ (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[67] Fig. 10 shows the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at three doses, $2E+11$, $2E+09$, and $2E+07$ (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[68] Fig. 11 shows that *Harryflintia acetispora* Strain A smEVs induce cytokine production from PMA-differentiated U937 cells. U937 cells were treated with *Harryflintia acetispora* Strain A smEV at 1×10^6 - 1×10^9 concentrations as well as TLR2 (FSL) and TLR4 (LPS) agonist controls for 24hrs and cytokine production was measured. Note the stepwise increase in cytokine production. "Blank" indicates the medium control.

[69] Fig. 12 shows that *Harryflintia acetispora* Strain A whole bacteria induce cytokine production from PMA-differentiated U937 cells. U937 cells were treated with *Harryflintia acetispora* Strain A whole gamma-irradiated microbes at 1×10^5 and 1×10^6 concentrations as well as TLR2 (FSL) and TLR4 (LPS) agonist controls for 24hrs and cytokine production was measured. "Blank" indicates the medium control.

[70] Fig. 13 shows the efficacy of *Harryflintia acetispora* Strain A smEVs and *Harryflintia acetispora* Strain A whole bacteria as compared to that of anti-PD-1 or vehicle in a mouse colorectal carcinoma model at day 22. Welch's test is performed for treatment vs. vehicle.

[71] Fig. 14 shows the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs to significantly reduce ear swelling at 24 hours post FITC ear challenge in a FITC-driven contact hypersensitivity model. Doses of $2E10$ and $2E7$ were tested. (ns: not significant)

[72] Fig. 15 shows the efficacy of orally administered *Megasphaera sp.* Strain A smEVs and *Harryflintia acetispora* Strain A smEVs to reduce ear swelling at 24 hours post FITC ear challenge in a FITC-driven contact hypersensitivity model. Doses of 2E9, 2E7 and 2E5 were tested. (ns: not significant).

[73] Figs. 16A-16E show the efficacy of orally administered *Megasphaera sp.* Strain A smEVs and *Harryflintia acetispora* Strain A smEVs to reduce mLN cytokine levels at 24 hours post FITC ear challenge in a FITC-driven contact hypersensitivity model. Doses of 2E9, 2E7 and 2E5 were tested. Compared to the vehicle group, *Harryflintia acetispora* Strain A smEVs and *Megasphaera sp.* Strain A smEVs treatment reduced cytokine levels for IL-4 (Fig. 16A), IL-5 (Fig. 16B), IL-13 (Fig. 16C), IL-10 (Fig. 16D), and IL-33 (Fig. 16E) in the mesenteric lymph nodes (mLNs)

[74] Fig. 17 shows the effects of blocking TLR2 signaling (with an anti-TLR2 antibody) on the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at a dose of 2E+11 (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[75] Fig. 18 shows the effects of blocking IL-10R signaling (with an anti-IL-10R antibody) on the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at a dose of 2E+11 (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[76] Fig. 19 shows the effects of blocking lymphocyte homing to the lymphoid tissue within the gut (with anti-CD62 and anti- $\alpha 4\beta 7$ antibodies) on the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at a dose of 2E+11 (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[77] Fig. 20 shows the effects of B cell depletion (with an anti-CD20 antibody) on the efficacy of orally administered *Harryflintia acetispora* Strain A smEVs at a dose of 2E+11 (based on particles per dose) in reducing antigen-specific ear swelling (ear thickness) at 24 hours compared to vehicle (negative control) and dexamethasone (positive control) following antigen challenge in a KLH-based delayed type hypersensitivity model.

[78] Fig. 21 shows that CD4+ T cells from mice dosed with *Harryflintia acetispora* Strain A smEVs at a dose of 2E+11 (based on particles per dose) were able to confer anti-inflammatory effects in recipient mice, as measured by reduced antigen-specific ear swelling (ear thickness) at 24 hours in recipient mice compared to vehicle (negative control) following antigen challenge in the recipient mice.

DETAILED DESCRIPTION

[79] The *Oscillospiraceae* family within the *Clostridia* class of microorganisms are common commensal organisms of vertebrates. Interestingly, despite being monoderm (usually described as Gram-positive) microbes they stain Gram-negative. Disclosed herein are investigations regarding whether *Harryflintia acetispora* could produce smEVs in sufficient yield to use commercially and found that relative to other Gram-positive organisms, members of this family produced high levels of smEVs, e.g., are high yield microbes. This defies the conventional understanding of smEV production within the field, as Gram-negative organisms typically produce higher numbers of smEVs. *Oscillospiraceae* family microbes, including *Harryflintia acetispora*, are generally difficult to both isolate and grow, thus previous investigations of smEVs from this family have been limited. From our investigations *Harryflintia acetispora* smEV elicits a unique cytokine response in *in vitro* coculture. smEVs from *Harryflintia acetispora* [e.g., *Harryflintia acetispora* Strain A] have demonstrated high efficacy and potency in animal models of inflammation and cancer. Provided herein are methods and compositions related to the use of *Harryflintia acetispora* bacteria and its derivatives (e.g., mEVs such as smEVs and/or pmEVs) in the treatment and/or prevention of a disease or a health disorder (e.g., adverse health disorders) (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease).

[80] High Yield Criterion for smEV Production: A crude mEV yield of 3×10^{13} per liter from a bioreactor-grown culture is the minimum threshold of the “high yield” designation for strains because this number was within one order of magnitude of the crude smEV yield of a reference strain that produces smEVs at a level that can reasonably be scaled for production.

Table 1 lists smEV yields from *Harryflintia acetispora* bacteria

Strain	Classification	Strain Name	EV Yield/ Liter
Strain A	Oscillospiraceae	<i>Harryflintia acetispora</i> S4-M5	3.20E+13

Definitions

[81] “Adjuvant” or “Adjuvant therapy” broadly refers to an agent that affects an immunological or physiological response in a patient or subject (e.g., human). For example, an adjuvant might increase the presence of an antigen over time or to an area of interest like a tumor, help absorb an antigen presenting cell antigen, activate macrophages and lymphocytes and support the production of cytokines. By changing an immune response, an adjuvant might permit a smaller dose of an immune interacting agent to increase the effectiveness or safety of a particular dose of the immune interacting agent. For example, an adjuvant might prevent T cell exhaustion and thus increase the effectiveness or safety of a particular immune interacting agent.

[82] “Administration” broadly refers to a route of administration of a composition (e.g., a pharmaceutical composition) to a subject. Examples of routes of administration include oral administration, rectal administration, topical administration, inhalation (nasal) or injection. Administration by injection includes intravenous (IV), intramuscular (IM), intratumoral (IT) and subcutaneous (SC) administration. A pharmaceutical composition described herein can be administered in any form by any effective route, including but not limited to intratumoral, oral, parenteral, enteral, intravenous, intraperitoneal, topical, transdermal (e.g., using any standard patch), intradermal, ophthalmic, (intra)nasally, local, non-oral, such as aerosol, inhalation, subcutaneous, intramuscular, buccal, sublingual, (trans)rectal, vaginal, intra-arterial, and intrathecal, transmucosal (e.g., sublingual, lingual, (trans)buccal, (trans)urethral, vaginal (e.g., trans- and perivaginally), implanted, intravesical, intrapulmonary, intraduodenal, intragastric, and intrabronchial. In preferred embodiments, a pharmaceutical composition described herein is administered orally, rectally, intratumorally, topically, intravesically, by injection into or adjacent to a draining lymph node, intravenously, by inhalation or aerosol, or subcutaneously. In another preferred embodiment, a pharmaceutical composition described herein is administered orally, intratumorally, or intravenously.

[83] As used herein, the term “antibody” may refer to both an intact antibody and an antigen binding fragment thereof. Intact antibodies are glycoproteins that include at least two

heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain includes a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain includes a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The term "antibody" includes, for example, monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, multispecific antibodies (e.g., bispecific antibodies), single-chain antibodies and antigen-binding antibody fragments.

[84] The terms "antigen binding fragment" and "antigen-binding portion" of an antibody, as used herein, refer to one or more fragments of an antibody that retain the ability to bind to an antigen. Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include Fab, Fab', F(ab')₂, Fv, scFv, disulfide linked Fv, Fd, diabodies, single-chain antibodies, NANOBODIES®, isolated CDRH3, and other antibody fragments that retain at least a portion of the variable region of an intact antibody. These antibody fragments can be obtained using conventional recombinant and/or enzymatic techniques and can be screened for antigen binding in the same manner as intact antibodies.

[85] "Cancer" broadly refers to an uncontrolled, abnormal growth of a host's own cells leading to invasion of surrounding tissue and potentially tissue distal to the initial site of abnormal cell growth in the host. Major classes include carcinomas which are cancers of the epithelial tissue (e.g., skin, squamous cells); sarcomas which are cancers of the connective tissue (e.g., bone, cartilage, fat, muscle, blood vessels, etc.); leukemias which are cancers of blood forming tissue (e.g., bone marrow tissue); lymphomas and myelomas which are cancers of immune cells; and central nervous system cancers which include cancers from brain and spinal tissue. "Cancer(s)" and "neoplasm(s)" are used herein interchangeably. As used herein, "cancer" refers to all types of cancer or neoplasm or malignant tumors including leukemias, carcinomas and sarcomas, whether new or recurring. Specific examples of cancers are: carcinomas, sarcomas, myelomas, leukemias, lymphomas and mixed type tumors. Non-limiting examples of cancers are new or recurring cancers of the brain, melanoma, bladder, breast, cervix, colon, head and neck, kidney, lung, non-small cell lung,

mesothelioma, ovary, prostate, sarcoma, stomach, uterus and medulloblastoma. In some embodiments, the cancer comprises a solid tumor. In some embodiments, the cancer comprises a metastasis.

[86] A “carbohydrate” refers to a sugar or polymer of sugars. The terms “saccharide,” “polysaccharide,” “carbohydrate,” and “oligosaccharide” may be used interchangeably. Most carbohydrates are aldehydes or ketones with many hydroxyl groups, usually one on each carbon atom of the molecule. Carbohydrates generally have the molecular formula $C_nH_{2n}O_n$. A carbohydrate may be a monosaccharide, a disaccharide, trisaccharide, oligosaccharide, or polysaccharide. The most basic carbohydrate is a monosaccharide, such as glucose, galactose, mannose, ribose, arabinose, xylose, and fructose. Disaccharides are two joined monosaccharides. Exemplary disaccharides include sucrose, maltose, cellobiose, and lactose. Typically, an oligosaccharide includes between three and six monosaccharide units (e.g., raffinose, stachyose), and polysaccharides include six or more monosaccharide units. Exemplary polysaccharides include starch, glycogen, and cellulose. Carbohydrates may contain modified saccharide units such as 2'-deoxyribose wherein a hydroxyl group is removed, 2'-fluororibose wherein a hydroxyl group is replaced with a fluorine, or N-acetylglucosamine, a nitrogen-containing form of glucose (e.g., 2'-fluororibose, deoxyribose, and hexose). Carbohydrates may exist in many different forms, for example, conformers, cyclic forms, acyclic forms, stereoisomers, tautomers, anomers, and isomers.

[87] “Cellular augmentation” broadly refers to the influx of cells or expansion of cells in an environment that are not substantially present in the environment prior to administration of a composition and not present in the composition itself. Cells that augment the environment include immune cells, stromal cells, bacterial and fungal cells. Environments of particular interest are the microenvironments where cancer cells reside or locate. In some instances, the microenvironment is a tumor microenvironment or a tumor draining lymph node. In other instances, the microenvironment is a pre-cancerous tissue site or the site of local administration of a composition or a site where the composition will accumulate after remote administration.

[88] “Clade” refers to the OTUs or members of a phylogenetic tree that are downstream of a statistically valid node in a phylogenetic tree. The clade comprises a set of terminal leaves in the phylogenetic tree that is a distinct monophyletic evolutionary unit and that share some extent of sequence similarity.

[89] A “combination” of mEVs (such as smEVs and/or pmEVs) from two or more microbial strains includes the physical co-existence of the microbes from which the mEVs (such as smEVs and/or pmEVs) are obtained, either in the same material or product or in physically connected products, as well as the temporal co-administration or co-localization of the mEVs (such as smEVs and/or pmEVs) from the two strains.

[90] The term “decrease” or “deplete” means a change, such that the difference is, depending on circumstances, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1/100, 1/1000, 1/10,000, 1/100,000, 1/1,000,000 or undetectable after treatment when compared to a pre-treatment state. Properties that may be decreased include the number of immune cells, bacterial cells, stromal cells, myeloid derived suppressor cells, fibroblasts, metabolites; the level of a cytokine; or another physical parameter (such as ear thickness (e.g., in a DTH animal model) or tumor size (e.g., in an animal tumor model)).

[91] “Dysbiosis” refers to a state of the microbiota or microbiome of the gut or other body area, including, e.g., mucosal or skin surfaces (or any other microbiome niche) in which the normal diversity and/or function of the host gut microbiome ecological networks (“microbiome”) are disrupted. A state of dysbiosis may result in a diseased state, or it may be unhealthy under only certain conditions or only if present for a prolonged period. Dysbiosis may be due to a variety of factors, including, environmental factors, infectious agents, host genotype, host diet and/or stress. A dysbiosis may result in: a change (e.g., increase or decrease) in the prevalence of one or more bacteria types (e.g., anaerobic), species and/or strains, change (e.g., increase or decrease) in diversity of the host microbiome population composition; a change (e.g., increase or reduction) of one or more populations of symbiont organisms resulting in a reduction or loss of one or more beneficial effects; overgrowth of one or more populations of pathogens (e.g., pathogenic bacteria); and/or the presence of, and/or overgrowth of, symbiotic organisms that cause disease only when certain conditions are present.

[92] The term “ecological consortium” is a group of bacteria which trades metabolites and positively co-regulates one another, in contrast to two bacteria which induce host synergy through activating complementary host pathways for improved efficacy.

[93] As used herein, “engineered bacteria” are any bacteria that have been genetically altered from their natural state by human activities, and the progeny of any such bacteria. Engineered bacteria include, for example, the products of targeted genetic modification, the products of random mutagenesis screens and the products of directed evolution.

[94] The term “epitope” means a protein determinant capable of specific binding to an antibody or T cell receptor. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains. Certain epitopes can be defined by a particular sequence of amino acids to which an antibody is capable of binding.

[95] The term “gene” is used broadly to refer to any nucleic acid associated with a biological function. The term “gene” applies to a specific genomic sequence, as well as to a cDNA or an mRNA encoded by that genomic sequence.

[96] “Identity” as between nucleic acid sequences of two nucleic acid molecules can be determined as a percentage of identity using known computer algorithms such as the “FASTA” program, using for example, the default parameters as in Pearson *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:2444 (other programs include the GCG program package (Devereux, J., *et al.*, Nucleic Acids Research 12(I):387 (1984)), BLASTP, BLASTN, FASTA Atschul, S. F., *et al.*, J Molec Biol 215:403 (1990); Guide to Huge Computers, Mrtin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo *et al.* (1988) SIAM J Applied Math 48:1073). For example, the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. Other commercially or publicly available programs include, DNASTar “MegAlign” program (Madison, Wis.) and the University of Wisconsin Genetics Computer Group (UWG) “Gap” program (Madison Wis.)).

[97] As used herein, the term “immune disorder” refers to any disease, disorder or disease symptom caused by an activity of the immune system, including autoimmune diseases, inflammatory diseases and allergies. Immune disorders include, but are not limited to, autoimmune diseases (e.g., psoriasis, atopic dermatitis, lupus, scleroderma, hemolytic anemia, vasculitis, type one diabetes, Grave’s disease, rheumatoid arthritis, multiple sclerosis, Goodpasture’s syndrome, pernicious anemia and/or myopathy), inflammatory diseases (e.g., acne vulgaris, asthma, celiac disease, chronic prostatitis, glomerulonephritis, inflammatory bowel disease, pelvic inflammatory disease, reperfusion injury, rheumatoid arthritis, sarcoidosis, transplant rejection, vasculitis and/or interstitial cystitis), and/or an allergies (e.g., food allergies, drug allergies and/or environmental allergies).

[98] “Immunotherapy” is treatment that uses a subject’s immune system to treat disease (e.g., immune disease, inflammatory disease, metabolic disease, cancer) and includes, for example, checkpoint inhibitors, cancer vaccines, cytokines, cell therapy, CAR-T cells, and dendritic cell therapy.

[99] The term “increase” means a change, such that the difference is, depending on circumstances, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 2-fold, 4-fold, 10-fold, 100-fold, 10^3 fold, 10^4 fold, 10^5 fold, 10^6 fold, and/or 10^7 fold greater after treatment when compared to a pre-treatment state. Properties that may be increased include the number of immune cells, bacterial cells, stromal cells, myeloid derived suppressor cells, fibroblasts, metabolites; the level of a cytokine; or another physical parameter (such as ear thickness (e.g., in a DTH animal model) or tumor size (e.g., in an animal tumor model).

[100] “Innate immune agonists” or “immuno-adjuvants” are small molecules, proteins, or other agents that specifically target innate immune receptors including Toll-Like Receptors (TLR), NOD receptors, RLRs, C-type lectin receptors, STING-cGAS Pathway components, inflammasome complexes. For example, LPS is a TLR-4 agonist that is bacterially derived or synthesized and aluminum can be used as an immune stimulating adjuvant. immuno-adjuvants are a specific class of broader adjuvant or adjuvant therapy. Examples of STING agonists include, but are not limited to, 2'3'-cGAMP, 3'3'-cGAMP, c-di-AMP, c-di-GMP, 2'2'-cGAMP, and 2'3'-cGAM(PS)₂ (Rp/Sp) (Rp, Sp-isomers of the bis-phosphorothioate analog of 2'3'-cGAMP). Examples of TLR agonists include, but are not limited to, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10 and TLR11. Examples of NOD agonists include, but are not limited to, N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide (MDP)), gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP), and desmuramylpeptides (DMP).

[101] The “internal transcribed spacer” or “ITS” is a piece of non-functional RNA located between structural ribosomal RNAs (rRNA) on a common precursor transcript often used for identification of eukaryotic species in particular fungi. The rRNA of fungi that forms the core of the ribosome is transcribed as a single gene and consists of the 8S, 5.8S and 28S regions with ITS4 and 5 between the 8S and 5.8S and 5.8S and 28S regions, respectively. These two intergenic segments between the 18S and 5.8S and 5.8S and 28S regions are removed by splicing and contain significant variation between species for barcoding purposes as previously described (Schoch et al Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109:6241-6246. 2012). 18S rDNA is traditionally used for phylogenetic reconstruction however the ITS can serve this function as it is generally highly conserved but contains hypervariable regions that harbor sufficient nucleotide diversity to differentiate genera and species of most fungus.

[102] The term “isolated” or “enriched” encompasses a microbe, an mEV (such as an smEV and/or pmEV) or other entity or substance that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature or in an experimental setting), and/or (2) produced, prepared, purified, and/or manufactured by the hand of man. Isolated microbes or mEVs (such as smEVs and/or pmEVs) may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated microbes or mEVs (such as smEVs and/or pmEVs) are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is “pure” if it is substantially free of other components. The terms “purify,” “purifying” and “purified” refer to a microbe or mEV or other material that has been separated from at least some of the components with which it was associated either when initially produced or generated (e.g., whether in nature or in an experimental setting), or during any time after its initial production. A microbe or a microbial population or mEV may be considered purified if it is isolated at or after production, such as from a material or environment containing the microbe or microbial population or mEV, and a purified microbe or microbial or mEV population may contain other materials up to about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or above about 90% and still be considered “isolated.” In some embodiments, purified microbes or mEVs (such as smEVs and/or pmEVs) or microbial population are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. In the instance of microbial compositions provided herein, the one or more microbial types present in the composition can be independently purified from one or more other microbes produced and/or present in the material or environment containing the microbial type. Microbial compositions and the microbial components such as mEVs (such as smEVs and/or pmEVs) thereof are generally purified from residual habitat products.

[103] As used herein a “lipid” includes fats, oils, triglycerides, cholesterol, phospholipids, fatty acids in any form including free fatty acids. Fats, oils and fatty acids can be saturated, unsaturated (cis or trans) or partially unsaturated (cis or trans).

[104] The term “LPS mutant or lipopolysaccharide mutant” broadly refers to selected bacteria that comprises loss of LPS. Loss of LPS might be due to mutations or disruption to genes involved in lipid A biosynthesis, such as *lpxA*, *lpxC*, and *lpxD*. Bacteria comprising LPS mutants can be resistant to aminoglycosides and polymyxins (polymyxin B and colistin).

[105] “Metabolite” as used herein refers to any and all molecular compounds, compositions, molecules, ions, co-factors, catalysts or nutrients used as substrates in any cellular or microbial metabolic reaction or resulting as product compounds, compositions, molecules, ions, co-factors, catalysts or nutrients from any cellular or microbial metabolic reaction.

[106] “Microbe” refers to any natural or engineered organism characterized as a archaeon, parasite, bacterium, fungus, microscopic alga, protozoan, and the stages of development or life cycle stages (e.g., vegetative, spore (including sporulation, dormancy, and germination), latent, biofilm) associated with the organism. Examples of gut microbes include: *Actinomyces graevenitzi*, *Actinomyces odontolyticus*, *Akkermansia muciniphila*, *Bacteroides caccae*, *Bacteroides fragilis*, *Bacteroides putredinis*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium wadsworthia*, *Blautia*, *Butyrivibrio*, *Campylobacter gracilis*, *Clostridia cluster III*, *Clostridia cluster IV*, *Clostridia cluster IX (Acidaminococcaceae group)*, *Clostridia cluster XI*, *Clostridia cluster XIII (Peptostreptococcus group)*, *Clostridia cluster XIV*, *Clostridia cluster XV*, *Collinsella aerofaciens*, *Coprococcus*, *Corynebacterium sunsvallense*, *Desulfomonas pigra*, *Dorea formicigenerans*, *Dorea longicatena*, *Escherichia coli*, *Eubacterium hadrum*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Gemella*, *Lactococcus*, *Lachnospira*, *Mollicutes cluster XVI*, *Mollicutes cluster XVIII*, *Prevotella*, *Rothia mucilaginosa*, *Ruminococcus callidus*, *Ruminococcus gnavus*, *Ruminococcus torques*, and *Streptococcus*.

[107] “Microbial extracellular vesicles” (mEVs) can be obtained from microbes such as bacteria, archaea, fungi, microscopic algae, protozoans, and parasites. In some embodiments, the mEVs (such as smEVs and/or pmEVs) are obtained from bacteria. mEVs include secreted microbial extracellular vesicles (smEVs) and processed microbial extracellular vesicles (pmEVs). “Secreted microbial extracellular vesicles” (smEVs) are naturally-produced vesicles derived from microbes. smEVs are comprised of microbial lipids and/or microbial proteins and/or microbial nucleic acids and/or microbial

carbohydrate moieties, and are isolated from culture supernatant. The natural production of these vesicles can be artificially enhanced (e.g., increased) or decreased through manipulation of the environment in which the bacterial cells are being cultured (e.g., by media or temperature alterations). Further, smEV compositions may be modified to reduce, increase, add, or remove microbial components or foreign substances to alter efficacy, immune stimulation, stability, immune stimulatory capacity, stability, organ targeting (e.g., lymph node), absorption (e.g., gastrointestinal), and/or yield (e.g., thereby altering the efficacy). As used herein, the term “purified smEV composition” or “smEV composition” refers to a preparation of smEVs that have been separated from at least one associated substance found in a source material (e.g., separated from at least one other microbial component) or any material associated with the smEVs in any process used to produce the preparation. It can also refer to a composition that has been significantly enriched for specific components. “Processed microbial extracellular vesicles” (pmEVs) are a non-naturally-occurring collection of microbial membrane components that have been purified from artificially lysed microbes (e.g., bacteria) (e.g., microbial membrane components that have been separated from other, intracellular microbial cell components), and which may comprise particles of a varied or a selected size range, depending on the method of purification. A pool of pmEVs is obtained by chemically disrupting (e.g., by lysozyme and/or lysostaphin) and/or physically disrupting (e.g., by mechanical force) microbial cells and separating the microbial membrane components from the intracellular components through centrifugation and/or ultracentrifugation, or other methods. The resulting pmEV mixture contains an enrichment of the microbial membranes and the components thereof (e.g., peripherally associated or integral membrane proteins, lipids, glycans, polysaccharides, carbohydrates, other polymers), such that there is an increased concentration of microbial membrane components, and a decreased concentration (e.g., dilution) of intracellular contents, relative to whole microbes. For gram-positive bacteria, pmEVs may include cell or cytoplasmic membranes. For gram-negative bacteria, a pmEV may include inner and outer membranes. pmEVs may be modified to increase purity, to adjust the size of particles in the composition, and/or modified to reduce, increase, add or remove, microbial components or foreign substances to alter efficacy, immune stimulation, stability, immune stimulatory capacity, stability, organ targeting (e.g., lymph node), absorption (e.g., gastrointestinal), and/or yield (e.g., thereby altering the efficacy). pmEVs can be modified by adding, removing, enriching for, or diluting specific components, including intracellular components

from the same or other microbes. As used herein, the term “purified pmEV composition” or “pmEV composition” refers to a preparation of pmEVs that have been separated from at least one associated substance found in a source material (e.g., separated from at least one other microbial component) or any material associated with the pmEVs in any process used to produce the preparation. It can also refer to a composition that has been significantly enriched for specific components.

[108] “Microbiome” broadly refers to the microbes residing on or in body site of a subject or patient. Microbes in a microbiome may include bacteria, viruses, eukaryotic microorganisms, and/or viruses. Individual microbes in a microbiome may be metabolically active, dormant, latent, or exist as spores, may exist planktonically or in biofilms, or may be present in the microbiome in sustainable or transient manner. The microbiome may be a commensal or healthy-state microbiome or a disease-state microbiome. The microbiome may be native to the subject or patient, or components of the microbiome may be modulated, introduced, or depleted due to changes in health state (e.g., precancerous or cancerous state) or treatment conditions (e.g., antibiotic treatment, exposure to different microbes). In some aspects, the microbiome occurs at a mucosal surface. In some aspects, the microbiome is a gut microbiome. In some aspects, the microbiome is a tumor microbiome.

[109] A “microbiome profile” or a “microbiome signature” of a tissue or sample refers to an at least partial characterization of the bacterial makeup of a microbiome. In some embodiments, a microbiome profile indicates whether at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more bacterial strains are present or absent in a microbiome. In some embodiments, a microbiome profile indicates whether at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more cancer-associated bacterial strains are present in a sample. In some embodiments, the microbiome profile indicates the relative or absolute amount of each bacterial strain detected in the sample. In some embodiments, the microbiome profile is a cancer-associated microbiome profile. A cancer-associated microbiome profile is a microbiome profile that occurs with greater frequency in a subject who has cancer than in the general population. In some embodiments, the cancer-associated microbiome profile comprises a greater number of or amount of cancer-associated bacteria than is normally present in a microbiome of an otherwise equivalent tissue or sample taken from an individual who does not have cancer.

[110] “Modified” in reference to a bacteria broadly refers to a bacteria that has undergone a change from its wild-type form. Bacterial modification can result from engineering bacteria. Examples of bacterial modifications include genetic modification, gene expression modification, phenotype modification, formulation modification, chemical modification, and dose or concentration. Examples of improved properties are described throughout this specification and include, e.g., attenuation, auxotrophy, homing, or antigenicity. Phenotype modification might include, by way of example, bacteria growth in media that modify the phenotype of a bacterium such that it increases or decreases virulence.

[111] An “oncobiome” as used herein comprises tumorigenic and/or cancer-associated microbiota, wherein the microbiota comprises one or more of a virus, a bacterium, a fungus, a protist, a parasite, or another microbe.

[112] “Oncotrophic” or “oncophilic” microbes and bacteria are microbes that are highly associated or present in a cancer microenvironment. They may be preferentially selected for within the environment, preferentially grow in a cancer microenvironment or hone to a said environment.

[113] “Operational taxonomic units” and “OTU(s)” refer to a terminal leaf in a phylogenetic tree and is defined by a nucleic acid sequence, e.g., the entire genome, or a specific genetic sequence, and all sequences that share sequence identity to this nucleic acid sequence at the level of species. In some embodiments the specific genetic sequence may be the 16S sequence or a portion of the 16S sequence. In other embodiments, the entire genomes of two entities are sequenced and compared. In another embodiment, select regions such as multilocus sequence tags (MLST), specific genes, or sets of genes may be genetically compared. For 16S, OTUs that share $\geq 97\%$ average nucleotide identity across the entire 16S or some variable region of the 16S are considered the same OTU. See e.g., Claesson MJ, Wang Q, O’Sullivan O, Greene-Diniz R, Cole JR, Ross RP, and O’Toole PW. 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res* 38: e200. Konstantinidis KT, Ramette A, and Tiedje JM. 2006. The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* 361: 1929–1940. For complete genomes, MLSTs, specific genes, other than 16S, or sets of genes OTUs that share $\geq 95\%$ average nucleotide identity are considered the same OTU. See e.g., Achtman M, and Wagner M. 2008. Microbial diversity and the genetic nature of microbial species. *Nat. Rev. Microbiol.* 6: 431–440. Konstantinidis KT, Ramette A, and Tiedje JM. 2006. The

bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* 361: 1929–1940. OTUs are frequently defined by comparing sequences between organisms. Generally, sequences with less than 95% sequence identity are not considered to form part of the same OTU. OTUs may also be characterized by any combination of nucleotide markers or genes, in particular highly conserved genes (e.g., “house-keeping” genes), or a combination thereof. Operational Taxonomic Units (OTUs) with taxonomic assignments made to, e.g., genus, species, and phylogenetic clade are provided herein.

[114] As used herein, a gene is “overexpressed” in a bacteria if it is expressed at a higher level in an engineered bacteria under at least some conditions than it is expressed by a wild-type bacteria of the same species under the same conditions. Similarly, a gene is “underexpressed” in a bacteria if it is expressed at a lower level in an engineered bacteria under at least some conditions than it is expressed by a wild-type bacteria of the same species under the same conditions.

[115] The terms “polynucleotide”, and “nucleic acid” are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), micro RNA (miRNA), silencing RNA (siRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. A polynucleotide may be further modified, such as by conjugation with a labeling component. In all nucleic acid sequences provided herein, U nucleotides are interchangeable with T nucleotides.

[116] As used herein, a substance is “pure” if it is substantially free of other components. The terms “purify,” “purifying” and “purified” refer to bacteria or an mEV (such as an smEV and/or a pmEV) preparation or other material that has been separated from at least some of the components with which it was associated either when initially produced or generated (e.g., whether in nature or in an experimental setting), or during any time after its initial production. An mEV (such as an smEV and/or a pmEV) preparation or

compositions may be considered purified if it is isolated at or after production, such as from one or more other bacterial components, and a purified microbe or microbial population may contain other materials up to about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or above about 90% and still be considered “purified.” In some embodiments, purified mEVs (such as smEVs and/or pmEVs) are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. mEV (such as an smEV and/or a pmEV) compositions (or preparations) are, e.g., purified from residual habitat products.

[117] As used herein, the term “purified mEV composition” or “mEV composition” refers to a preparation that includes mEVs (such as smEVs and/or pmEVs) that have been separated from at least one associated substance found in a source material (e.g., separated from at least one other bacterial component) or any material associated with the mEVs (such as smEVs and/or pmEVs) in any process used to produce the preparation. It also refers to a composition that has been significantly enriched or concentrated. In some embodiments, the mEVs (such as smEVs and/or pmEVs) are concentrated by 2 fold, 3-fold, 4-fold, 5-fold, 10-fold, 100-fold, 1000-fold, 10,000-fold or more than 10,000 fold.

[118] “Residual habitat products” refers to material derived from the habitat for microbiota within or on a subject. For example, fermentation cultures of microbes can contain contaminants, e.g., other microbe strains or forms (e.g., bacteria, virus, mycoplasma, and/or fungus). For example, microbes live in feces in the gastrointestinal tract, on the skin itself, in saliva, mucus of the respiratory tract, or secretions of the genitourinary tract (i.e., biological matter associated with the microbial community). Substantially free of residual habitat products means that the microbial composition no longer contains the biological matter associated with the microbial environment on or in the culture or human or animal subject and is 100% free, 99% free, 98% free, 97% free, 96% free, or 95% free of any contaminating biological matter associated with the microbial community. Residual habitat products can include abiotic materials (including undigested food) or it can include unwanted microorganisms. Substantially free of residual habitat products may also mean that the microbial composition contains no detectable cells from a culture contaminant or a human or animal and that only microbial cells are detectable. In one embodiment, substantially free of residual habitat products may also mean that the microbial composition contains no detectable viral (including bacteria, viruses (e.g., phage)), fungal, mycoplasmal

contaminants. In another embodiment, it means that fewer than $1 \times 10^{-2}\%$, $1 \times 10^{-3}\%$, $1 \times 10^{-4}\%$, $1 \times 10^{-5}\%$, $1 \times 10^{-6}\%$, $1 \times 10^{-7}\%$, $1 \times 10^{-8}\%$ of the viable cells in the microbial composition are human or animal, as compared to microbial cells. There are multiple ways to accomplish this degree of purity, none of which are limiting. Thus, contamination may be reduced by isolating desired constituents through multiple steps of streaking to single colonies on solid media until replicate (such as, but not limited to, two) streaks from serial single colonies have shown only a single colony morphology. Alternatively, reduction of contamination can be accomplished by multiple rounds of serial dilutions to single desired cells (e.g., a dilution of 10^{-8} or 10^{-9}), such as through multiple 10-fold serial dilutions. This can further be confirmed by showing that multiple isolated colonies have similar cell shapes and Gram staining behavior. Other methods for confirming adequate purity include genetic analysis (e.g., PCR, DNA sequencing), serology and antigen analysis, enzymatic and metabolic analysis, and methods using instrumentation such as flow cytometry with reagents that distinguish desired constituents from contaminants.

[119] As used herein, “specific binding” refers to the ability of an antibody to bind to a predetermined antigen or the ability of a polypeptide to bind to its predetermined binding partner. Typically, an antibody or polypeptide specifically binds to its predetermined antigen or binding partner with an affinity corresponding to a K_D of about 10^{-7} M or less, and binds to the predetermined antigen/binding partner with an affinity (as expressed by K_D) that is at least 10 fold less, at least 100 fold less or at least 1000 fold less than its affinity for binding to a non-specific and unrelated antigen/binding partner (e.g., BSA, casein). Alternatively, specific binding applies more broadly to a two component system where one component is a protein, lipid, or carbohydrate or combination thereof and engages with the second component which is a protein, lipid, carbohydrate or combination thereof in a specific way.

[120] “Strain” refers to a member of a bacterial species with a genetic signature such that it may be differentiated from closely-related members of the same bacterial species. The genetic signature may be the absence of all or part of at least one gene, the absence of all or part of at least one regulatory region (e.g., a promoter, a terminator, a riboswitch, a ribosome binding site), the absence (“curing”) of at least one native plasmid, the presence of at least one recombinant gene, the presence of at least one mutated gene, the presence of at least one foreign gene (a gene derived from another species), the presence at least one mutated regulatory region (e.g., a promoter, a terminator, a riboswitch, a ribosome

binding site), the presence of at least one non-native plasmid, the presence of at least one antibiotic resistance cassette, or a combination thereof. Genetic signatures between different strains may be identified by PCR amplification optionally followed by DNA sequencing of the genomic region(s) of interest or of the whole genome. In the case in which one strain (compared with another of the same species) has gained or lost antibiotic resistance or gained or lost a biosynthetic capability (such as an auxotrophic strain), strains may be differentiated by selection or counter-selection using an antibiotic or nutrient/metabolite, respectively.

[121] The terms “subject” or “patient” refers to any mammal. A subject or a patient described as “in need thereof” refers to one in need of a treatment (or prevention) for a disease. Mammals (i.e., mammalian animals) include humans, laboratory animals (e.g., primates, rats, mice), livestock (e.g., cows, sheep, goats, pigs), and household pets (e.g., dogs, cats, rodents). The subject may be a human. The subject may be a non-human mammal including but not limited to of a dog, a cat, a cow, a horse, a pig, a donkey, a goat, a camel, a mouse, a rat, a guinea pig, a sheep, a llama, a monkey, a gorilla or a chimpanzee. The subject may be healthy, or may be suffering from a cancer at any developmental stage, wherein any of the stages are either caused by or opportunistically supported of a cancer associated or causative pathogen, or may be at risk of developing a cancer, or transmitting to others a cancer associated or cancer causative pathogen. In some embodiments, a subject has lung cancer, bladder cancer, prostate cancer, plasmacytoma, colorectal cancer, rectal cancer, Merkel Cell carcinoma, salivary gland carcinoma, ovarian cancer, and/or melanoma. The subject may have a tumor. The subject may have a tumor that shows enhanced macropinocytosis with the underlying genomics of this process including Ras activation. In other embodiments, the subject has another cancer. In some embodiments, the subject has undergone a cancer therapy.

[122] As used herein, the term “treating” a disease in a subject or “treating” a subject having or suspected of having a disease refers to administering to the subject to a pharmaceutical treatment, e.g., the administration of one or more agents, such that at least one symptom of the disease is decreased or prevented from worsening. Thus, in one embodiment, “treating” refers inter alia to delaying progression, expediting remission, inducing remission, augmenting remission, speeding recovery, increasing efficacy of or decreasing resistance to alternative therapeutics, or a combination thereof. As used herein, the term “preventing” a disease in a subject refers to administering to the subject to a

pharmaceutical treatment, e.g., the administration of one or more agents, such that onset of at least one symptom of the disease is delayed or prevented.

[123] As used herein, a "type" of bacteria may be distinguished from other bacteria by: genus, species, sub-species, strain or by any other taxonomic categorization, whether based on morphology, physiology, genotype, protein expression or other characteristics known in the art.

Bacteria

[124] In certain aspects, provided herein are pharmaceutical compositions that comprise mEVs (such as smEVs and/or pmEVs) from *Harryflintia acetispora* bacteria, *Harryflintia acetispora* bacteria, or any combination thereof. In some embodiments the *Harryflintia acetispora* bacteria are high yield strains of *Harryflintia acetispora* bacteria. In some embodiments, the high yield strain produces at least 3×10^{13} mEVs per liter from a bioreactor-grown culture.

[125] In some embodiments, the *Harryflintia acetispora* bacteria are modified to reduce toxicity or other adverse effects, to enhance delivery) (e.g., oral delivery) of the mEVs (such as smEVs and/or pmEVs), bacteria for pharmaceutical compositions, or any combination thereof, (e.g., by improving acid resistance, muco-adherence and/or penetration and/or resistance to bile acids, digestive enzymes, resistance to anti-microbial peptides and/or antibody neutralization), to target desired cell types (e.g., M-cells, goblet cells, enterocytes, dendritic cells, macrophages), to enhance their immunomodulatory and/or therapeutic effect of the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof (e.g., either alone or in combination with another therapeutic agent), and/or to enhance immune activation or suppression by the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof (e.g., through modified production of polysaccharides, pili, fimbriae, adhesins). In some embodiments, the engineered *Harryflintia acetispora* bacteria described herein are modified to improve manufacturing mEVs (such as smEVs and/or pmEVs), bacteria for pharmaceutical compositions, or any combination thereof (e.g., higher oxygen tolerance, stability, improved freeze-thaw tolerance, shorter generation times). For example, in some embodiments, the engineered *Harryflintia acetispora* bacteria described include bacteria harboring one or more genetic changes, such change being an insertion, deletion, translocation, or substitution, or any combination thereof, of one or more nucleotides contained on the bacterial chromosome or endogenous plasmid and/or one or

more foreign plasmids, wherein the genetic change may result in the overexpression and/or underexpression of one or more genes. The engineered *Harryflintia acetispora* bacteria may be produced using any technique known in the art, including but not limited to site-directed mutagenesis, transposon mutagenesis, knock-outs, knock-ins, polymerase chain reaction mutagenesis, chemical mutagenesis, ultraviolet light mutagenesis, transformation (chemically or by electroporation), phage transduction, directed evolution, or any combination thereof.

[126] In some embodiments, the *Harryflintia acetispora* bacterial strain is a bacterial strain having a genome that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to a strain listed in Table 2.

[127] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, described herein are obtained from a strain of *Harryflintia acetispora* bacteria comprising a genomic sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity (e.g., at least 99.5% sequence identity, at least 99.6% sequence identity, at least 99.7% sequence identity, at least 99.8% sequence identity, at least 99.9% sequence identity) to the genomic sequence of the strain of bacteria deposited with the ATCC Deposit number as provided in Table 2. In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, described herein are obtained from a strain of *Harryflintia acetispora* bacteria comprising a 16S sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity (e.g., at least 99.5% sequence identity, at least 99.6% sequence identity, at least 99.7% sequence identity, at least 99.8% sequence identity, at least 99.9% sequence identity) to the 16S sequence as provided in Table 2.

[128] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are lyophilized.

[129] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are gamma irradiated (e.g., at 17.5 or 25 kGy).

[130] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are UV irradiated.

[131] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are heat inactivated (e.g., at 50°C for two hours or at 90°C for two hours).

[132] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are acid treated.

[133] In some embodiments, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, of the pharmaceutical compositions described herein are oxygen sparged (e.g., at 0.1 vvm for two hours).

[134] The phase of growth can affect the amount or properties of bacteria and/or mEVs (such as smEVs and/or pmEVs) produced by bacteria. For example, in the methods of bacteria preparation provided herein, bacteria can be isolated, e.g., from a culture, at the start of the log phase of growth, midway through the log phase, and/or once stationary phase growth has been reached. As another example, in the methods of preparing mEVs (such as smEVs and/or pmEVs) provided herein, mEVs (such as smEVs and/or pmEVs) can be prepared from a culture, at the start of the log phase of growth, midway through the log phase, and/or once stationary phase growth has been reached.

Table 2: Exemplary *Harryflintia acetispora* Bacterial Strain

SEQ ID NO	Strain	Deposit Number	16S Ribosomal RNA Sequence
SEQ ID NO: 1	<i>Harryflintia acetispora</i> strain A	PTA-126694	CTAAAGAGTTTGATCCTGGCTCAGGA CGAACGCTGGCGGCGCGCTAACACA TGCAAGTCGAACGGAGAAATGCTGAG CTTGCTTTGCATTTCTTAGTGGCGGAC GGGTGAGTAACACGTGAGCAACCTGC CTTTGTGAGGGGAATAACGTCTGGAA ACGGACGCTAATACCGCATAACGTCA AGGAACCGCATGGTTTTTTGACCAAA GATTTTATCGCACAAAGATGGGCTCG CGGCTGATTAGCTAGTTGGCGGGTA ACGGCCCCACCAAGGCGACGATCAGTA GCCGGACTGAGAGGTTGATCGGCCAC ATTGGGACTGAGACACGGCCCAGACT CCTACGGGAGGCAGCAGTGGGGGATA

		TTGCACAATGGGGGAAACCCTGATGC AGCGACGCCGCGTGAGGGAAGACGGT TTTCGGATTGTAAACCTCTGTCTTCAG GGACGAAATCAATGACGGTACCTGAG GAGGAAGCCACGGCTAACTACGTGCC AGCAGCCGCGGTAATACGTAGGTGGC AAGCGTTGTCCGGAATTACTGGGTGT AAAGGGAGCGTAGGCGGGAATGCAA GTTGAATGTTTAACTATCGGCTCAAC TGATAATCGCGTTCAAACTGCATTTC TTGAGTGGAGTAGAGGCAGGCGGAAT TCCTAGTGTAGCGGTGAAATGCGTAG ATATTAGGAGGAACACCAGTGGCGAA GGCGGCCTGCTGGGCTCTAACTGACG CTGAGGCTCGAAAGCGTGGGTAGCAA ACAGGATTAGATAACCCTGGTAGTCCA CGCCGTAAACGATGATTACTAGGTGT GGGGGGACTGACCCCTTCCGTGCCGG AGTTAACACAATAAGTAATCCACCTG GGGAGTACGGTCGCAAGACTGAAACT CAAAGGAATTGACGGGGGCCCCGCACA AGCAGTGGAGTATGTGGTTTAAATTG AAGCAACGCGAAGAACCTTACCAGGT CTTGACATCGTGCGCATACCGTAGAG ATACGGGAAGTCCTTCGGGACGCATA GACAGGTGGTGCATGGTTGTCGTCAG CTCGTGTCGTGAGATGTTGGGTAAAGT CCCACAACGAGCGCAACCCTTATTAT TAGTTGCTACGCAAGAGCACTCTAAT GAGACTGCCGTTGACAAAACGGAGGA AGGTGGGGATGACGTCAAATCATCAT GCCCCCTTATGACCTGGGCTACACACG TACTACAATGGCACTTAACAGAGGGA AGCAAGACCGCGAGGTGGAGCAAAC CCCCAAAAGTGTCTCAGTTCGGATT GCAGGCTGCAACCCGCCTGTATGAAG TCGGAATTGCTAGTAATCGCGGATCA GCATGCCGCGGTGAATACGTTCCCGG GCCTTGACACACCGCCCGTCACACC ATGAGAGCCGGAACACCCGAAGTCA GTAGCCTAACCGCAAGGAGGGCGCTG CCGAAGGTGGGATTGGTGATTAGGGT GAAGTCGTAAACAAGGTAGCCGTATCG GAAGGTGCGGCTGGATCACCTCCTTT
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[135] Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, the *Harryflintia*

acetispora Strain A was deposited on March 5, 2020, with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Va. 20110-2209 USA and was assigned ATCC Accession Number PTA-126694.

[136] Applicant represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited plasmid, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicant acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Modified mEVs

[137] In some aspects, the mEVs (such as smEVs and/or pmEVs) described herein are modified such that they comprise, are linked to, and/or are bound by a therapeutic moiety.

[138] In some embodiments, the therapeutic moiety is a cancer-specific moiety. In some embodiments, the cancer-specific moiety has binding specificity for a cancer cell (e.g., has binding specificity for a cancer-specific antigen). In some embodiments, the cancer-specific moiety comprises an antibody or antigen binding fragment thereof. In some embodiments, the cancer-specific moiety comprises a T cell receptor or a chimeric antigen receptor (CAR). In some embodiments, the cancer-specific moiety comprises a ligand for a receptor expressed on the surface of a cancer cell or a receptor-binding fragment thereof. In some embodiments, the cancer-specific moiety is a bipartite fusion protein that has two parts: a first part that binds to and/or is linked to the bacterium and a second part that is capable of binding to a cancer cell (e.g., by having binding specificity for a cancer-specific antigen). In some embodiments, the first part is a fragment of or a full-length peptidoglycan recognition protein, such as PGRP. In some embodiments the first part has binding specificity for the mEV (e.g., by having binding specificity for a bacterial antigen). In some

embodiments, the first and/or second part comprises an antibody or antigen binding fragment thereof. In some embodiments, the first and/or second part comprises a T cell receptor or a chimeric antigen receptor (CAR). In some embodiments, the first and/or second part comprises a ligand for a receptor expressed on the surface of a cancer cell or a receptor-binding fragment thereof. In certain embodiments, co-administration of the cancer-specific moiety with the mEVs (either in combination or in separate administrations) increases the targeting of the mEVs to the cancer cells.

[139] In some embodiments, the mEVs described herein are modified such that they comprise, are linked to, and/or are bound by a magnetic and/or paramagnetic moiety (e.g., a magnetic bead). In some embodiments, the magnetic and/or paramagnetic moiety is comprised by and/or directly linked to the bacteria. In some embodiments, the magnetic and/or paramagnetic moiety is linked to and/or a part of an mEV-binding moiety that binds to the mEV. In some embodiments, the mEV-binding moiety is a fragment of or a full-length peptidoglycan recognition protein, such as PGRP. In some embodiments the mEV-binding moiety has binding specificity for the mEV (e.g., by having binding specificity for a bacterial antigen). In some embodiments, the mEV-binding moiety comprises an antibody or antigen binding fragment thereof. In some embodiments, the mEV-binding moiety comprises a T cell receptor or a chimeric antigen receptor (CAR). In some embodiments, the mEV-binding moiety comprises a ligand for a receptor expressed on the surface of a cancer cell or a receptor-binding fragment thereof. In certain embodiments, co-administration of the magnetic and/or paramagnetic moiety with the mEVs (either together or in separate administrations) can be used to increase the targeting of the mEVs (e.g., to cancer cells and/or a part of a subject where cancer cells are present).

Production of Processed Microbial Extracellular Vesicles (pmEVs)

[140] In certain aspects, the pmEVs described herein can be prepared using any method known in the art.

[141] In some embodiments, the pmEVs are prepared without a pmEV purification step. For example, in some embodiments, *Harryflintia acetispora* bacteria from which the pmEVs described herein are released are killed using a method that leaves the *Harryflintia acetispora* bacterial pmEVs intact, and the resulting *Harryflintia acetispora* bacterial components, including the pmEVs, are used in the methods and compositions described herein. In some embodiments, the *Harryflintia acetispora* bacteria are killed using an

antibiotic (e.g., using an antibiotic described herein). In some embodiments, the *Harryflintia acetispora* bacteria are killed using UV irradiation.

[142] In some embodiments, the pmEVs described herein are purified from one or more other *Harryflintia acetispora* bacterial components. Methods for purifying pmEVs from *Harryflintia acetispora* bacteria (and optionally, other bacterial components) are known in the art. In some embodiments, pmEVs are prepared from *Harryflintia acetispora* bacterial cultures using methods described in Thein, *et al.* (*J. Proteome Res.* 9(12):6135-6147 (2010)) or Sandrini, *et al.* (*Bio-protocol* 4(21): e1287 (2014)), each of which is hereby incorporated by reference in its entirety. In some embodiments, the bacteria are cultured to high optical density and then centrifuged to pellet bacteria (e.g., at 10,000- 15,000 x g for 10- 15 min at room temperature or 4°C). In some embodiments, the supernatants are discarded and cell pellets are frozen at -80°C. In some embodiments, cell pellets are thawed on ice and resuspended in 100 mM Tris-HCl, pH 7.5 supplemented with 1 mg/mL DNase I. In some embodiments, cells are lysed using an Emulsiflex C-3 (Avestin, Inc.) under conditions recommended by the manufacturer. In some embodiments, debris and unlysed cells are pelleted by centrifugation at 10,000 x g for 15 min at 4°C. In some embodiments, supernatants are then centrifuged at 120,000 x g for 1 hour at 4°C. In some embodiments, pellets are resuspended in ice-cold 100 mM sodium carbonate, pH 11, incubated with agitation for 1 hr at 4°C, and then centrifuged at 120,000 x g for 1 hour at 4°C. In some embodiments, pellets are resuspended in 100 mM Tris-HCl, pH 7.5, re-centrifuged at 120,000 x g for 20 min at 4°C, and then resuspended in 0.1 M Tris-HCl, pH 7.5 or in PBS. In some embodiments, samples are stored at -20°C.

[143] In certain aspects, pmEVs are obtained by methods adapted from Sandrini *et al.*, 2014. In some embodiments, *Harryflintia acetispora* bacterial cultures are centrifuged at 10,000-15,500 x g for 10-15 min at room temp or at 4°C. In some embodiments, cell pellets are frozen at -80°C and supernatants are discarded. In some embodiments, cell pellets are thawed on ice and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA supplemented with 0.1 mg/mL lysozyme. In some embodiments, samples are incubated with mixing at room temp or at 37°C for 30 min. In some embodiments, samples are re-frozen at -80°C and thawed again on ice. In some embodiments, DNase I is added to a final concentration of 1.6 mg/mL and MgCl₂ to a final concentration of 100 mM. In some embodiments, samples are sonicated using a QSonica Q500 sonicator with 7 cycles of 30 sec on and 30 sec off. In some embodiments, debris and unlysed cells are pelleted by centrifugation at 10,000 x g for 15

min. at 4°C. In some embodiments, supernatants are then centrifuged at 110,000 x g for 15 min at 4°C. In some embodiments, pellets are resuspended in 10 mM Tris-HCl, pH 8.0, 2% Triton X-100 and incubated 30-60 min with mixing at room temperature. In some embodiments, samples are centrifuged at 110,000 x g for 15 min at 4°C. In some embodiments, pellets are resuspended in PBS and stored at -20°C.

[144] In certain aspects, a method of forming (e.g., preparing) isolated *Harryflintia acetispora* bacterial pmEVs, described herein, comprises the steps of: (a) centrifuging a *Harryflintia acetispora* bacterial culture, thereby forming a first pellet and a first supernatant, wherein the first pellet comprises cells; (b) discarding the first supernatant; (c) resuspending the first pellet in a solution; (d) lysing the cells; (e) centrifuging the lysed cells, thereby forming a second pellet and a second supernatant; (f) discarding the second pellet and centrifuging the second supernatant, thereby forming a third pellet and a third supernatant; (g) discarding the third supernatant and resuspending the third pellet in a second solution, thereby forming the isolated *Harryflintia acetispora* bacterial pmEVs.

[145] In some embodiments, the method further comprises the steps of: (h) centrifuging the solution of step (g), thereby forming a fourth pellet and a fourth supernatant; (i) discarding the fourth supernatant and resuspending the fourth pellet in a third solution. In some embodiments, the method further comprises the steps of: (j) centrifuging the solution of step (i), thereby forming a fifth pellet and a fifth supernatant; and (k) discarding the fifth supernatant and resuspending the fifth pellet in a fourth solution.

[146] In some embodiments, the centrifugation of step (a) is at 10,000 x g. In some embodiments the centrifugation of step (a) is for 10-15 minutes. In some embodiments, the centrifugation of step (a) is at 4 °C or room temperature. In some embodiments, step (b) further comprises freezing the first pellet at -80 °C . In some embodiments, the solution in step (c) is 100mM Tris-HCl, pH 7.5 supplemented with 1mg/ml DNaseI. In some embodiments, the solution in step (c) is 10mM Tris-HCl, pH 8.0, 1mM EDTA, supplemented with 0.1 mg/ml lysozyme. In some embodiments, step (c) further comprises incubating for 30 minutes at 37 °C or room temperature. In some embodiments, step (c) further comprises freezing the first pellet at -80 °C . In some embodiments, step (c) further comprises adding DNase I to a final concentration of 1.6mg/ml. In some embodiments, step (c) further comprises adding MgCl₂ to a final concentration of 100mM. In some embodiments, the cells are lysed in step (d) via homogenization. In some embodiments, the cells are lysed in step (d) via emulsiflex C3. In some embodiments, the cells are lysed in step

(d) via sonication. In some embodiments, the cells are sonicated in 7 cycles, wherein each cycle comprises 30 seconds of sonication and 30 seconds without sonication. In some embodiments, the centrifugation of step (e) is at 10,000 x g. In some embodiments, the centrifugation of step (e) is for 15 minutes. In some embodiments, the centrifugation of step (e) is at 4 °C or room temperature.

[147] In some embodiments, the centrifugation of step (f) is at 120,000 x g. In some embodiments, the centrifugation of step (f) is at 110,000 x g. In some embodiments, the centrifugation of step (f) is for 1 hour. In some embodiments, the centrifugation of step (f) is for 15 minutes. In some embodiments, the centrifugation of step (f) is at 4 °C or room temperature. In some embodiments, the second solution in step (g) is 100 mM sodium carbonate, pH 11. In some embodiments, the second solution in step (g) is 10mM Tris-HCl pH 8.0, 2% triton X-100. In some embodiments, step (g) further comprises incubating the solution for 1 hour at 4 °C. In some embodiments, step (g) further comprises incubating the solution for 30-60 minutes at room temperature. In some embodiments, the centrifugation of step (h) is at 120,000 x g. In some embodiments, the centrifugation of step (h) is at 110,000 x g. In some embodiments, the centrifugation of step (h) is for 1 hour. In some embodiments, the centrifugation of step (h) is for 15 minutes. In some embodiments, the centrifugation of step (h) is at 4 °C or room temperature. In some embodiments, the third solution in step (i) is 100mM Tris-HCl, pH 7.5. In some embodiments, the third solution in step (i) is PBS. In some embodiments, the centrifugation of step (j) is at 120,000 x g. In some embodiments, the centrifugation of step (j) is for 20 minutes. In some embodiments, the centrifugation of step (j) is at 4 °C or room temperature. In some embodiments, the fourth solution in step (k) is 100mM Tris-HCl, pH 7.5 or PBS.

[148] pmEVs obtained by methods provided herein may be further purified by size based column chromatography, by affinity chromatography, and by gradient ultracentrifugation, using methods that may include, but are not limited to, use of a sucrose gradient or Optiprep gradient. Briefly, using a sucrose gradient method, if ammonium sulfate precipitation or ultracentrifugation were used to concentrate the filtered supernatants, pellets are resuspended in 60% sucrose, 30 mM Tris, pH 8.0. If filtration was used to concentrate the filtered supernatant, the concentrate is buffer exchanged into 60% sucrose, 30 mM Tris, pH 8.0, using an Amicon Ultra column. Samples are applied to a 35-60% discontinuous sucrose gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C. Briefly, using an Optiprep gradient method, if ammonium sulfate precipitation or ultracentrifugation were

used to concentrate the filtered supernatants, pellets are resuspended in 35% Optiprep in PBS. In some embodiments, if filtration was used to concentrate the filtered supernatant, the concentrate is diluted using 60% Optiprep to a final concentration of 35% Optiprep. Samples are applied to a 35-60% discontinuous sucrose gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C.

[149] In some embodiments, to confirm sterility and isolation of the pmEV preparations, pmEVs are serially diluted onto agar medium used for routine culture of the bacteria being tested, and incubated using routine conditions. Non-sterile preparations are passed through a 0.22 µm filter to exclude intact cells. To further increase purity, isolated pmEVs may be DNase or proteinase K treated.

[150] In some embodiments, the sterility of the pmEV preparations can be confirmed by plating a portion of the pmEVs onto agar medium used for standard culture of the bacteria used in the generation of the pmEVs and incubating using standard conditions.

[151] In some embodiments select pmEVs are isolated and enriched by chromatography and binding surface moieties on pmEVs. In other embodiments, select pmEVs are isolated and/or enriched by fluorescent cell sorting by methods using affinity reagents, chemical dyes, recombinant proteins or other methods known to one skilled in the art.

[152] The pmEVs can be analyzed, e.g., as described in Jeppesen, et al. Cell 177:428 (2019).

[153] In some embodiments, pmEVs are lyophilized. In some embodiments, pmEVs are gamma irradiated (e.g., at 17.5 or 25 kGy). In some embodiments, pmEVs are UV irradiated. In some embodiments, pmEVs are heat inactivated (e.g., at 50°C for two hours or at 90°C for two hours). In some embodiments, pmEVs are acid treated. In some embodiments, pmEVs are oxygen sparged (e.g., at 0.1 vvm for two hours).

[154] The phase of growth can affect the amount or properties of bacteria. In the methods of pmEV preparation provided herein, pmEVs can be isolated, e.g., from a culture, at the start of the log phase of growth, midway through the log phase, and/or once stationary phase growth has been reached.

Production of Secreted Microbial Extracellular Vesicles (smEVs)

[155] In certain aspects, the smEVs described herein can be prepared using any method known in the art.

[156] In some embodiments, the smEVs are prepared without an smEV purification step. For example, in some embodiments, bacteria described herein are killed using a method that leaves the smEVs intact and the resulting *Harryflintia acetispora* bacterial components, including the smEVs, are used in the methods and compositions described herein. In some embodiments, the *Harryflintia acetispora* bacteria are killed using an antibiotic (e.g., using an antibiotic described herein). In some embodiments, the *Harryflintia acetispora* bacteria are killed using UV irradiation. In some embodiments, the *Harryflintia acetispora* bacteria are heat-killed.

[157] In some embodiments, the smEVs described herein are purified from one or more other *Harryflintia acetispora* bacterial components. Methods for purifying smEVs from bacteria are known in the art. In some embodiments, smEVs are prepared from *Harryflintia acetispora* bacterial cultures using methods described in S. Bin Park, et al. PLoS ONE. 6(3):e17629 (2011) or G. Norheim, et al. PLoS ONE. 10(9): e0134353 (2015) or Jeppesen, et al. Cell 177:428 (2019), each of which is hereby incorporated by reference in its entirety. In some embodiments, the *Harryflintia acetispora* bacteria are cultured to high optical density and then centrifuged to pellet *Harryflintia acetispora* bacteria (e.g., at 10,000 x g for 30 min at 4°C, at 15,500 x g for 15 min at 4°C). In some embodiments, the culture supernatants are then passed through filters to exclude intact bacterial cells (e.g., a 0.22 µm filter). In some embodiments, the supernatants are then subjected to tangential flow filtration, during which the supernatant is concentrated, species smaller than 100 kDa are removed, and the media is partially exchanged with PBS. In some embodiments, filtered supernatants are centrifuged to pellet bacterial smEVs (e.g., at 100,000-150,000 x g for 1-3 hours at 4°C, at 200,000 x g for 1-3 hours at 4°C). In some embodiments, the smEVs are further purified by resuspending the resulting smEV pellets (e.g., in PBS), and applying the resuspended smEVs to an Optiprep (iodixanol) gradient or gradient (e.g., a 30-60% discontinuous gradient, a 0-45% discontinuous gradient), followed by centrifugation (e.g., at 200,000 x g for 4-20 hours at 4°C). smEV bands can be collected, diluted with PBS, and centrifuged to pellet the smEVs (e.g., at 150,000 x g for 3 hours at 4°C, at 200,000 x g for 1 hour at 4°C). The purified smEVs can be stored, for example, at -80°C or -20°C until use. In

some embodiments, the smEVs are further purified by treatment with DNase and/or proteinase K.

[158] For example, in some embodiments, cultures of *Harryflintia acetispora* bacteria can be centrifuged at 11,000 x g for 20-40 min at 4°C to pellet bacteria. Culture supernatants may be passed through a 0.22 µm filter to exclude intact bacterial cells. Filtered supernatants may then be concentrated using methods that may include, but are not limited to, ammonium sulfate precipitation, ultracentrifugation, or filtration. For example, for ammonium sulfate precipitation, 1.5-3 M ammonium sulfate can be added to filtered supernatant slowly, while stirring at 4°C. Precipitations can be incubated at 4°C for 8-48 hours and then centrifuged at 11,000 x g for 20-40 min at 4°C. The resulting pellets contain bacteria smEVs and other debris. Using ultracentrifugation, filtered supernatants can be centrifuged at 100,000-200,000 x g for 1-16 hours at 4°C. The pellet of this centrifugation contains bacteria smEVs and other debris such as large protein complexes. In some embodiments, using a filtration technique, such as through the use of an Amicon Ultra spin filter or by tangential flow filtration, supernatants can be filtered so as to retain species of molecular weight > 50 or 100 kDa.

[159] Alternatively, smEVs can be obtained from *Harryflintia acetispora* bacteria cultures continuously during growth, or at selected time points during growth, for example, by connecting a bioreactor to an alternating tangential flow (ATF) system (e.g., XCell ATF from Repligen). The ATF system retains intact cells (>0.22 µm) in the bioreactor, and allows smaller components (e.g., smEVs, free proteins) to pass through a filter for collection. For example, the system may be configured so that the <0.22 µm filtrate is then passed through a second filter of 100 kDa, allowing species such as smEVs between 0.22 µm and 100 kDa to be collected, and species smaller than 100 kDa to be pumped back into the bioreactor. Alternatively, the system may be configured to allow for medium in the bioreactor to be replenished and/or modified during growth of the culture. smEVs collected by this method may be further purified and/or concentrated by ultracentrifugation or filtration as described above for filtered supernatants.

[160] smEVs obtained by methods provided herein may be further purified by size-based column chromatography, by affinity chromatography, by ion-exchange chromatography, and by gradient ultracentrifugation, using methods that may include, but are not limited to, use of a sucrose gradient or Optiprep gradient. Briefly, using a sucrose gradient method, if ammonium sulfate precipitation or ultracentrifugation were used to

concentrate the filtered supernatants, pellets are resuspended in 60% sucrose, 30 mM Tris, pH 8.0. If filtration was used to concentrate the filtered supernatant, the concentrate is buffer exchanged into 60% sucrose, 30 mM Tris, pH 8.0, using an Amicon Ultra column. Samples are applied to a 35-60% discontinuous sucrose gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C. Briefly, using an Optiprep gradient method, if ammonium sulfate precipitation or ultracentrifugation were used to concentrate the filtered supernatants, pellets are resuspended in PBS and 3 volumes of 60% Optiprep are added to the sample. In some embodiments, if filtration was used to concentrate the filtered supernatant, the concentrate is diluted using 60% Optiprep to a final concentration of 35% Optiprep. Samples are applied to a 0-45% discontinuous Optiprep gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C, e.g., 4-24 hours at 4°C.

[161] In some embodiments, to confirm sterility and isolation of the smEV preparations, smEVs are serially diluted onto agar medium used for routine culture of the bacteria being tested, and incubated using routine conditions. Non-sterile preparations are passed through a 0.22 µm filter to exclude intact cells. To further increase purity, isolated smEVs may be DNase or proteinase K treated.

[162] In some embodiments, for preparation of smEVs used for in vivo injections, purified smEVs are processed as described previously (G. Norheim, et al. PLoS ONE. 10(9): e0134353 (2015)). Briefly, after sucrose gradient centrifugation, bands containing smEVs are resuspended to a final concentration of 50 µg/mL in a solution containing 3% sucrose or other solution suitable for in vivo injection known to one skilled in the art. This solution may also contain adjuvant, for example aluminum hydroxide at a concentration of 0-0.5% (w/v). In some embodiments, for preparation of smEVs used for in vivo injections, smEVs in PBS are sterile-filtered to < 0.22 µm.

[163] In certain embodiments, to make samples compatible with further testing (e.g., to remove sucrose prior to TEM imaging or in vitro assays), samples are buffer exchanged into PBS or 30 mM Tris, pH 8.0 using filtration (e.g., Amicon Ultra columns), dialysis, or ultracentrifugation (200,000 x g, ≥ 3 hours, 4°C) and resuspension.

[164] In some embodiments, the sterility of the smEV preparations can be confirmed by plating a portion of the smEVs onto agar medium used for standard culture of the bacteria used in the generation of the smEVs and incubating using standard conditions.

[165] In some embodiments, select smEVs are isolated and enriched by chromatography and binding surface moieties on smEVs. In other embodiments, select

smEVs are isolated and/or enriched by fluorescent cell sorting by methods using affinity reagents, chemical dyes, recombinant proteins or other methods known to one skilled in the art.

[166] The smEVs can be analyzed, e.g., as described in Jeppesen, et al. Cell 177:428 (2019).

[167] In some embodiments, smEVs are lyophilized. In some embodiments, smEVs are gamma irradiated (e.g., at 17.5 or 25 kGy). In some embodiments, smEVs are UV irradiated. In some embodiments, smEVs are heat inactivated (e.g., at 50°C for two hours or at 90°C for two hours). In some embodiments, smEVs are acid treated. In some embodiments, smEVs are oxygen sparged (e.g., at 0.1 vvm for two hours).

[168] The phase of growth can affect the amount or properties of *Harryflintia acetispora* bacteria and/or smEVs produced by *Harryflintia acetispora* bacteria. For example, in the methods of smEV preparation provided herein, smEVs can be isolated, e.g., from a culture, at the start of the log phase of growth, midway through the log phase, and/or once stationary phase growth has been reached.

[169] The growth environment (e.g., culture conditions) can affect the amount of smEVs produced by *Harryflintia acetispora* bacteria. For example, the yield of smEVs can be increased by an smEV inducer, as provided in Table 3.

Table 3: Culture Techniques to Increase smEV Production

smEV inducement	smEV inducer	Acts on
Temperature		
	Heat RT to 37 °C temp change 37 to 40 °C temp change	stress response simulates infection febrile infection
ROS		
	Plumbagin Cumene hydroperoxide Hydrogen Peroxide	oxidative stress response oxidative stress response oxidative stress response
Antibiotics		
	Ciprofloxacin Gentamycin Polymyxin B D-cycloserine	bacterial SOS response protein synthesis outer membrane cell wall
Osmolyte		
	NaCl	osmotic stress
Metal Ion Stress		

	Iron Chelation EDTA Low Hemin	iron levels removes divalent cations iron levels
Media additives or removal		
	Lactate Amino acid deprivation Hexadecane Glucose Sodium bicarbonate PQS Diamines+ DFMO High nutrients Low nutrients	growth stress stress growth ToxT induction vesiculator (from bacteria) membrane anchoring (negativicutes only) enhanced growth
Other mechanisms	Oxygen No Cysteine Inducing biofilm or flocculation Diauxic Growth Phage Urea	oxygen stress in anaerobe oxygen stress in anaerobe

[170] In the methods of smEVs preparation provided herein, the method can optionally include exposing a culture of *Harryflintia acetispora* bacteria to an smEV inducer prior to isolating smEVs from the bacterial culture. The culture of *Harryflintia acetispora* bacteria can be exposed to an smEV inducer at the start of the log phase of growth, midway through the log phase, and/or once stationary phase growth has been reached.

Pharmaceutical Compositions

[171] In certain aspects, provided herein are pharmaceutical compositions (e.g., bacterial formulations or bacterial compositions) that comprise mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, obtained from *Harryflintia acetispora* bacteria.

[172] In certain aspects, provided herein are pharmaceutical compositions comprising *Harryflintia acetispora* described herein and a pharmaceutically acceptable carrier.

3.2x10¹¹, 3.3x10¹¹, 3.4x10¹¹, 3.5x10¹¹, 3.6x10¹¹, 3.7x10¹¹, 3.8x10¹¹, 3.9x10¹¹, 4x10¹¹ 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, 9x10¹¹, 1x10¹², 1.5x10¹² colony forming units (CFU) of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A).

[176] In some embodiments, the pharmaceutical composition comprises about 1 x 10⁵, 5 x 10⁵, 1 x 10⁶, 2 x 10⁶, 3 x 10⁶, 4 x 10⁶, 5 x 10⁶, 6 x 10⁶, 7 x 10⁶, 8 x 10⁶, 9 x 10⁶, 1 x 10⁷, 2 x 10⁷, 3 x 10⁷, 4 x 10⁷, 5 x 10⁷, 6 x 10⁷, 7 x 10⁷, 8 x 10⁷, 9 x 10⁷, 1 x 10⁸, 2 x 10⁸, 3 x 10⁸, 4 x 10⁸, 5 x 10⁸, 6 x 10⁸, 7 x 10⁸, 8 x 10⁸, 9 x 10⁸ or 1 x 10⁹, 1 x 10¹⁰, 2x10¹⁰, 2.1x10¹⁰, 2.2x10¹⁰, 2.3x10¹⁰, 2.4x10¹⁰, 2.5x10¹⁰, 2.6x10¹⁰, 2.7x10¹⁰, 2.8x10¹⁰, 2.9x10¹⁰, 3x10¹⁰, 3.1x10¹⁰, 3.2x10¹⁰, 3.3x10¹⁰, 3.4x10¹⁰, 3.5x10¹⁰, 3.6x10¹⁰, 3.7x10¹⁰, 3.8x10¹⁰, 3.9x10¹⁰, 4x10¹⁰, 5x10¹⁰, 6x10¹⁰, 7x10¹⁰, 8x10¹⁰, 9x10¹⁰, 1x10¹¹, 1.1x10¹¹, 1.2x10¹¹, 1.3x10¹¹, 1.4x10¹¹, 1.5x10¹¹, 1.6x10¹¹, 1.7x10¹¹, 1.8x10¹¹, 1.9x10¹¹, 2x10¹¹, 2.1x10¹¹, 2.2x10¹¹, 2.3x10¹¹, 2.4x10¹¹, 2.5x10¹¹, 2.6x10¹¹, 2.7x10¹¹, 2.8x10¹¹, 2.9x10¹¹, 3x10¹¹, 3.1x10¹¹, 3.2x10¹¹, 3.3x10¹¹, 3.4x10¹¹, 3.5x10¹¹, 3.6x10¹¹, 3.7x10¹¹, 3.8x10¹¹, 3.9x10¹¹, 4x10¹¹ 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, 9x10¹¹, 1x10¹², 1.5x10¹² total cells of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A).

[177] In some embodiments, the pharmaceutical composition comprises at least 1 x 10⁵, 5 x 10⁵, 1 x 10⁶, 2 x 10⁶, 3 x 10⁶, 4 x 10⁶, 5 x 10⁶, 6 x 10⁶, 7 x 10⁶, 8 x 10⁶, 9 x 10⁶, 1 x 10⁷, 2 x 10⁷, 3 x 10⁷, 4 x 10⁷, 5 x 10⁷, 6 x 10⁷, 7 x 10⁷, 8 x 10⁷, 9 x 10⁷, 1 x 10⁸, 2 x 10⁸, 3 x 10⁸, 4 x 10⁸, 5 x 10⁸, 6 x 10⁸, 7 x 10⁸, 8 x 10⁸, 9 x 10⁸ or 1 x 10⁹, 1 x 10¹⁰, 2x10¹⁰, 2.1x10¹⁰, 2.2x10¹⁰, 2.3x10¹⁰, 2.4x10¹⁰, 2.5x10¹⁰, 2.6x10¹⁰, 2.7x10¹⁰, 2.8x10¹⁰, 2.9x10¹⁰, 3x10¹⁰, 3.1x10¹⁰, 3.2x10¹⁰, 3.3x10¹⁰, 3.4x10¹⁰, 3.5x10¹⁰, 3.6x10¹⁰, 3.7x10¹⁰, 3.8x10¹⁰, 3.9x10¹⁰, 4x10¹⁰, 5x10¹⁰, 6x10¹⁰, 7x10¹⁰, 8x10¹⁰, 9x10¹⁰, 1x10¹¹, 1.1x10¹¹, 1.2x10¹¹, 1.3x10¹¹, 1.4x10¹¹, 1.5x10¹¹, 1.6x10¹¹, 1.7x10¹¹, 1.8x10¹¹, 1.9x10¹¹, 2x10¹¹, 2.1x10¹¹, 2.2x10¹¹, 2.3x10¹¹, 2.4x10¹¹, 2.5x10¹¹, 2.6x10¹¹, 2.7x10¹¹, 2.8x10¹¹, 2.9x10¹¹, 3x10¹¹, 3.1x10¹¹, 3.2x10¹¹, 3.3x10¹¹, 3.4x10¹¹, 3.5x10¹¹, 3.6x10¹¹, 3.7x10¹¹, 3.8x10¹¹, 3.9x10¹¹, 4x10¹¹ 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, 9x10¹¹, 1x10¹², 1.5x10¹² total cells of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A).

[178] In some embodiments, the pharmaceutical composition comprises at most 1 x 10⁵, 5 x 10⁵, 1 x 10⁶, 2 x 10⁶, 3 x 10⁶, 4 x 10⁶, 5 x 10⁶, 6 x 10⁶, 7 x 10⁶, 8 x 10⁶, 9 x 10⁶, 1 x 10⁷, 2 x 10⁷, 3 x 10⁷, 4 x 10⁷, 5 x 10⁷, 6 x 10⁷, 7 x 10⁷, 8 x 10⁷, 9 x 10⁷, 1 x 10⁸, 2 x 10⁸, 3 x 10⁸, 4 x 10⁸, 5 x 10⁸, 6 x 10⁸, 7 x 10⁸, 8 x 10⁸, 9 x 10⁸ or 1 x 10⁹, 1 x 10¹⁰, 2x10¹⁰, 2.1x10¹⁰, 2.2x10¹⁰, 2.3x10¹⁰, 2.4x10¹⁰, 2.5x10¹⁰, 2.6x10¹⁰, 2.7x10¹⁰, 2.8x10¹⁰, 2.9x10¹⁰, 3x10¹⁰, 3.1x10¹⁰, 3.2x10¹⁰, 3.3x10¹⁰, 3.4x10¹⁰, 3.5x10¹⁰, 3.6x10¹⁰, 3.7x10¹⁰, 3.8x10¹⁰, 3.9x10¹⁰,

4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 1.1×10^{11} , 1.2×10^{11} , 1.3×10^{11} , 1.4×10^{11} , 1.5×10^{11} , 1.6×10^{11} , 1.7×10^{11} , 1.8×10^{11} , 1.9×10^{11} , 2×10^{11} , 2.1×10^{11} , 2.2×10^{11} , 2.3×10^{11} , 2.4×10^{11} , 2.5×10^{11} , 2.6×10^{11} , 2.7×10^{11} , 2.8×10^{11} , 2.9×10^{11} , 3×10^{11} , 3.1×10^{11} , 3.2×10^{11} , 3.3×10^{11} , 3.4×10^{11} , 3.5×10^{11} , 3.6×10^{11} , 3.7×10^{11} , 3.8×10^{11} , 3.9×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , 1×10^{12} , 1.5×10^{12} total cells of *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A).

[179] In certain embodiments, the pharmaceutical composition can include a total protein amount of at least 5 mg (e.g., at least 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100 mg, 110 mg, 120 mg, 130 mg, 140 mg, 150 mg, 160 mg, 170 mg, 180 mg, 190 mg, 200 mg, 210 mg, 220 mg, 230 mg, 240 mg, 250 mg, 260 mg, 270 mg, 280 mg, 290 mg, 300 mg, 310 mg, 320 mg, 330 mg, 340 mg, 350 mg, 360 mg, 370 mg, 380 mg, 390 mg, 400 mg, 410 mg, 420 mg, 430 mg, 440 mg, 450 mg, 460 mg, 470 mg, 480 mg, 490 mg, 500 mg, 510 mg, 520 mg, 530 mg, 540 mg, 550 mg, 560 mg, 570 mg, 580 mg, 590 mg, 600 mg, 610 mg, 620 mg, 630 mg, 640 mg, 650 mg, 660 mg, 670 mg, 680 mg, 690 mg, 700 mg, 710 mg, 720 mg, 730 mg, 740 mg, 750 mg, 760 mg, 770 mg, 780 mg, 790 mg, 800 mg, 810 mg, 820 mg, 830 mg, 840 mg, 850 mg, 860 mg, 870 mg, 880 mg, 890 mg, or 900 mg,) and no more than 900 mg (e.g., no more than 890 mg, 880 mg, 870 mg, 860 mg, 850 mg, 840 mg, 830 mg, 820 mg, 810 mg, 800 mg, 790 mg, 780 mg, 770 mg, 760 mg, 750 mg, 740 mg, 730 mg, 720 mg, 710 mg, 700 mg, 690 mg, 680 mg, 670 mg, 660 mg, 650 mg, 640 mg, 630 mg, 620 mg, 610 mg, 600 mg, 590 mg, 580 mg, 570 mg, 560 mg, 550 mg, 540 mg, 530 mg, 520 mg, 510 mg, 500 mg, 490 mg, 480 mg, 470 mg, 460 mg, 450 mg, 440 mg, 430 mg, 420 mg, 410 mg, 400 mg, 390 mg, 380 mg, 370 mg, 360 mg, 350 mg, 340 mg, 330 mg, 320 mg, 310 mg, 300 mg, 290 mg, 280 mg, 270 mg, 260 mg, 250 mg, 240 mg, 230 mg, 220 mg, 210 mg, 200 mg, 190 mg, 180 mg, 170 mg, 160 mg, 150 mg, 140 mg, 130 mg, 120 mg, 110 mg, 100 mg, 90 mg, 80 mg, 70 mg, 60 mg, 50 mg, 40 mg, 30 mg, 20 mg, or 10 mg) (e.g., as determined by a Bradford assay, as determined by a BCA assay). In certain embodiments, the pharmaceutical composition can include a total protein amount of about 10 mg, about 20 mg, about 30 mg, about 40 mg, about 50 mg, about 60 mg, about 70 mg, about 80 mg, about 90 mg, about 100 mg, about 110 mg, about 120 mg, about 130 mg, about 140 mg, about 150 mg, about 160 mg, about 170 mg, about 180 mg, about 190 mg, about 200 mg, about 210 mg, about 220 mg, about 230 mg, about 240 mg, about 250 mg, about 260 mg, about 270 mg, about 280 mg, about 290 mg, about 300 mg, about 310 mg, about 320 mg, about 330 mg, about 340 mg, about 350 mg, about 360 mg, about 370 mg, about 380 mg, about 390

mg, about 400 mg, about 410 mg, about 420 mg, about 430 mg, about 440 mg, about 450 mg, about 460 mg, about 470 mg, about 480 mg, about 490 mg, about 500 mg, about 510 mg, about 520 mg, about 530 mg, about 540 mg, about 550 mg, about 560 mg, about 570 mg, about 580 mg, about 590 mg, about 600 mg, about 610 mg, about 620 mg, about 630 mg, about 640 mg, about 650 mg, about 660 mg, about 670 mg, about 680 mg, about 690 mg, about 700 mg, about 710 mg, about 720 mg, about 730 mg, about 740 mg, about 750 mg, about 760 mg, about 770 mg, about 780 mg, about 790 mg, about 800 mg, about 810 mg, about 820 mg, about 830 mg, about 840 mg, about 850 mg, about 860 mg, about 870 mg, about 880 mg, about 890 mg, or about 900 mg (*e.g.*, as determined by a Bradford assay, or as determined by a BCA assay).

[180] In some embodiments, the pharmaceutical composition can include a total amount of *Harryflintia acetispora* bacteria of at least 5 mg (*e.g.*, at least 6 mg, at least 7 mg, at least 8 mg, at least 9 mg, at least 10 mg, at least 11 mg, at least 12 mg, at least 13 mg, at least 14 mg, at least 15 mg, at least 16 mg, at least 17 mg, at least 18 mg, at least 19 mg, or at least 20 mg) and no more than 20 mg (*e.g.*, no more than 19 mg, no more than 18 mg, no more than 17 mg, no more than 16 mg, no more than 15 mg, no more than 14 mg, no more than 13 mg, no more than 12 mg, no more than 11 mg, no more than 10 mg, no more than 9 mg, no more than 8 mg, no more than 7 mg, no more than 6 mg, no more than 5 mg) (*e.g.*, as determined by a Bradford assay, or as determined by a BCA assay). In some embodiments, the pharmaceutical composition can include a total amount of *Harryflintia acetispora* bacteria of about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, about 10 mg, about 11 mg, about 12 mg, about 13 mg, about 14 mg, about 15 mg, about 16 mg, about 17 mg, about 18 mg, about 19 mg, or about 20 mg (*e.g.*, as determined by a Bradford assay, or as determined by a BCA assay).

[181] In certain embodiments, the pharmaceutical composition (*e.g.*, composition of the total dose administered, *e.g.*, once or twice daily) comprises at least 1×10^{10} total cells (*e.g.*, at least 1×10^{10} total cells, at least 2×10^{10} total cells, at least 3×10^{10} total cells, at least 4×10^{10} total cells, at least 5×10^{10} total cells, at least 6×10^{10} total cells, at least 7×10^{10} total cells, at least 8×10^{10} total cells, at least 9×10^{10} total cells, at least 1×10^{11} total cells) of the *Harryflintia acetispora* bacteria. In some embodiments, the pharmaceutical composition comprises no more than 9×10^{11} total cells (*e.g.*, no more than 1×10^{10} total cells, no more than 2×10^{10} total cells, no more than 3×10^{10} total cells, no more than 4×10^{10} total cells, no more than 5×10^{10} total cells, no more than 6×10^{10} total cells, no more

than 7×10^{10} total cells, no more than 8×10^{10} total cells, no more than 9×10^{10} total cells, no more than 1×10^{11} total cells, no more than 2×10^{11} total cells, no more than 3×10^{11} total cells, no more than 4×10^{11} total cells, no more than 5×10^{11} total cells, no more than 6×10^{11} total cells, no more than 7×10^{11} total cells, no more than 8×10^{11} total cells) of the *Harryflintia acetispora* bacteria. In some embodiments, the pharmaceutical composition comprises about 6×10^9 total cells of the *Harryflintia acetispora* bacteria.

[182] In some embodiments, the bacterial and/or pharmaceutical composition comprises live, killed, attenuated, lyophilized, and/or irradiated (e.g., UV or gamma irradiated) bacteria. Bacteria may be heat-killed by pasteurization, sterilization, high temperature treatment, spray cooking and/or spray drying (heat treatments can be performed at 50°C, 65°C, 85°C or a variety of other temperatures and/or a varied amount of time). Bacteria may also be killed or inactivated using γ -irradiation (gamma irradiation), exposure to UV light, formalin-inactivation, and/or freezing methods, or a combination thereof. For example, the bacteria may be exposed to 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, or 50kGy of radiation prior to administration. In some embodiments, bacteria are killed using gamma irradiation. In some embodiments, the bacteria are killed or inactivated using electron irradiation (e.g., beta radiation) or x-ray irradiation.

[183] In some embodiments, the bacteria in the bacterial and/or pharmaceutical composition described herein are killed using a method that leaves the disease modulating activity of the bacteria intact and the resulting bacterial components are used in the methods and compositions described herein. In some embodiments, the bacteria in the composition described herein are killed using an antibiotic (e.g., using an antibiotic described herein). In some embodiments, the bacteria in the composition described herein are killed using UV irradiation.

[184] In some embodiments, the bacteria in the composition described herein are killed using heat (temperature) sterilization, filtration, and radiation using methods known to those skilled in the art (Garg M., see the World Wide Web at biologydiscussion.com/microorganisms/sterilization/top-3-physical-methods-used-to-kill-microorganisms/55243). The bacteria may be killed via E-beam using methods known to those skilled in the art (SİLİNDİR M. et al, FABAD J. Pharm. Sci., 34, 43–53, 2009). In some embodiments, the bacteria in the composition described herein are killed and/or attenuated by a chemical agent, for example, aldehydes, e.g., formaldehyde, glutaraldehyde, and the like; food preservative agents such as SO₂, sorbic acid, benzoic acid, nitrate, and

nitrite salts; gases such as ethylene oxide; halogens, such as iodine, chlorine, and the like; peroxygens, such as ozone, peroxide, peracetic acid; bisphenols; phenols; phenolics; biguanides, e.g., chlorhexidine; and the like.

[185] Bacteria may be grown to various growth phases and tested for efficacy at different dilutions and at different points during the growth phase. For example, bacteria may be tested for efficacy following administration at stationary phase (including early or late stationary phase), or at various timepoints during exponential phase. In addition to inactivation by various methods, bacteria may be tested for efficacy using different ratios of live versus inactivated cells, or different ratios of cells at various growth phases.

[186] In certain embodiments, provided herein are pharmaceutical compositions comprising mEVs (such as smEVs and/or pmEVs) (e.g., an mEV composition (e.g., an smEV composition or a pmEV composition)) from *Harryflintia acetispora* (e.g., *Harryflintia acetispora* Strain A). In some embodiments, the mEV composition comprises mEVs (such as smEVs and/or pmEVs) and/or a combination of mEVs (such as smEVs and/or pmEVs) described herein and a pharmaceutically acceptable carrier. In some embodiments, the smEV composition comprises smEVs and/or a combination of smEVs described herein and a pharmaceutically acceptable carrier. In some embodiments, the pmEV composition comprises pmEVs and/or a combination of pmEVs described herein and a pharmaceutically acceptable carrier.

[187] In some embodiments, the pharmaceutical compositions comprise mEVs (such as smEVs and/or pmEVs) substantially or entirely free of whole bacteria (e.g., live bacteria, killed bacteria, attenuated bacteria). In some embodiments, the pharmaceutical compositions comprise both mEVs and whole bacteria (e.g., live bacteria, killed bacteria, attenuated bacteria). In some embodiments, the pharmaceutical composition comprises lyophilized mEVs (such as smEVs and/or pmEVs). In some embodiments, the pharmaceutical composition comprises gamma irradiated mEVs (such as smEVs and/or pmEVs). The mEVs (such as smEVs and/or pmEVs) can be gamma irradiated after the mEVs are isolated (e.g., prepared). In some embodiments, the pharmaceutical compositions comprise mEVs from *Harryflintia acetispora* Strain A.

[188] In some embodiments, to quantify the numbers of mEVs (such as smEVs and/or pmEVs) and/or bacteria present in a bacterial sample, electron microscopy (e.g., EM of ultrathin frozen sections) can be used to visualize the mEVs (such as smEVs and/or pmEVs) and/or bacteria and count their relative numbers. Alternatively, nanoparticle

tracking analysis (NTA), Coulter counting, or dynamic light scattering (DLS) or a combination of these techniques can be used. NTA and the Coulter counter count particles and show their sizes. DLS gives the size distribution of particles, but not the concentration. Bacteria frequently have diameters of 1-2 μm (microns). The full range is 0.2-20 μm . Combined results from Coulter counting and NTA can reveal the numbers of bacteria and/or mEVs (such as smEVs and/or pmEVs) in a given sample. Coulter counting reveals the numbers of particles with diameters of 0.7-10 μm . For most bacterial and/or mEV (such as smEV and/or pmEV) samples, the Coulter counter alone can reveal the number of bacteria and/or mEVs (such as smEVs and/or pmEVs) in a sample. pmEVs are 20-600 nm in diameter. For NTA, a Nanosight instrument can be obtained from Malvern Pananalytical. For example, the NS300 can visualize and measure particles in suspension in the size range 10-2000nm. NTA allows for counting of the numbers of particles that are, for example, 50-1000 nm in diameter. DLS reveals the distribution of particles of different diameters within an approximate range of 1 nm – 3 μm .

[189] mEVs can be characterized by analytical methods known in the art (e.g., Jeppesen, et al. Cell 177:428 (2019)).

[190] In some embodiments, the mEVs (such as smEVs and/or pmEVs) may be quantified based on particle count. For example, particle count of an mEV preparation can be measured using NTA.

[191] In some embodiments, the mEVs (such as smEVs and/or pmEVs) may be quantified based on the amount of protein, lipid, or carbohydrate. For example, total protein content of an mEV preparation can be measured using the Bradford assay or BCA assay.

[192] In some embodiments, the mEVs (such as smEVs and/or pmEVs) are isolated away from one or more other bacterial components of the source bacteria. In some embodiments, the pharmaceutical composition further comprises other bacterial components.

[193] In certain embodiments, the mEV preparation obtained from the source bacteria may be fractionated into subpopulations based on the physical properties (e.g., sized, density, protein content, binding affinity) of the subpopulations. One or more of the mEV subpopulations can then be incorporated into the pharmaceutical compositions of the invention.

[194] In some embodiments, the *Harryflintia acetispora* bacteria may be quantified based on particle count. For example, total particle content of a *Harryflintia acetispora* bacteria can be measured using NTA.

[195] In some embodiments, the *Harryflintia acetispora* bacteria may be quantified based on total cell count (TCC) (e.g., determined by Coulter counter).

[196] In some embodiments, the *Harryflintia acetispora* bacteria may be quantified using a plate count assay (e.g., by creating serial dilutions of the bacteria, allowing them to grow on a suitable medium, and then counting the number of colonies).

[197] In some embodiments, the *Harryflintia acetispora* bacteria may be quantified based on the amount of protein, lipid, or carbohydrate. For example, total protein content of a *Harryflintia acetispora* bacteria preparation can be measured using the Bradford assay or the BCA assay.

[198] For oral administration to a human subject, the dose of *Harryflintia acetispora* bacteria can be, e.g., about 2×10^6 - about 2×10^{16} particles. The dose can be, e.g., about 1×10^7 - about 1×10^{15} , about 1×10^8 - about 1×10^{14} , about 1×10^9 - about 1×10^{13} , about 1×10^{10} - about 1×10^{14} , or about 1×10^8 - about 1×10^{12} particles. The dose can be, e.g., about 2×10^6 , about 2×10^7 , about 2×10^8 , about 2×10^9 , about 1×10^{10} , about 2×10^{10} , about 2×10^{11} , about 2×10^{12} , about 2×10^{13} , about 2×10^{14} , or about 1×10^{15} particles. The dose can be, e.g., about 2×10^{14} particles. The dose can be, e.g., about 2×10^{12} particles. The dose can be, e.g., about 2×10^{10} particles. The dose can be, e.g., about 1×10^{10} particles. Particle count can be determined, e.g., by NTA.

[199] For oral administration to a human subject, the dose of *Harryflintia acetispora* bacteria can be, e.g., based on total protein. The dose can be, e.g., about 5 mg to about 900 mg total protein. The dose can be, e.g., about 20 mg to about 800 mg, about 50 mg to about 700 mg, about 75 mg to about 600 mg, about 100 mg to about 500 mg, about 250 mg to about 750 mg, or about 200 mg to about 500 mg total protein. The dose can be, e.g., about 10 mg, about 25 mg, about 50 mg, about 75 mg, about 100 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, or about 750 mg total protein. The dose can be, e.g., about 10 mg total protein. Total protein can be determined, e.g., by Bradford assay or by the BCA assay.

[200] For administration by injection (e.g., intravenous administration) to a human subject, the dose of *Harryflintia acetispora* bacteria can be, e.g., about 1×10^6 - about 1×10^{16} particles. The dose can be, e.g., about 1×10^7 - about 1×10^{15} , about 1×10^8 - about 1×10^{14} , about

1×10^9 - about 1×10^{13} , about 1×10^{10} - about 1×10^{14} , or about 1×10^8 - about 1×10^{12} particles. The dose can be, e.g., about 2×10^6 , about 2×10^7 , about 2×10^8 , about 2×10^9 , about 1×10^{10} , about 2×10^{10} , about 2×10^{11} , about 2×10^{12} , about 2×10^{13} , about 2×10^{14} , or about 1×10^{15} particles. The dose can be, e.g., about 1×10^{15} particles. The dose can be, e.g., about 2×10^{14} particles. The dose can be, e.g., about 2×10^{13} particles. Particle count can be determined, e.g., by NTA.

[201] For administration by injection (e.g., intravenous administration), the dose of *Harryflintia acetispora* bacteria can be, e.g., about 5 mg to about 900 mg total protein. The dose can be, e.g., about 20 mg to about 800 mg, about 50 mg to about 700 mg, about 75 mg to about 600 mg, about 100 mg to about 500 mg, about 250 mg to about 750 mg, or about 200 mg to about 500 mg total protein. The dose can be, e.g., about 10 mg, about 25 mg, about 50 mg, about 75 mg, about 100 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, or about 750 mg total protein. The dose can be, e.g., about 700 mg total protein. The dose can be, e.g., about 350 mg total protein. The dose can be, e.g., about 175 mg total protein. Total protein can be determined, e.g., by Bradford assay or by the BCA assay.

[202] In certain embodiments, the pharmaceutical composition (e.g., composition of the total dose administered, e.g., once or twice daily) comprises at least 1×10^{10} total cells (e.g., at least 1×10^{10} total cells, at least 2×10^{10} total cells, at least 3×10^{10} total cells, at least 4×10^{10} total cells, at least 5×10^{10} total cells, at least 6×10^{10} total cells, at least 7×10^{10} total cells, at least 8×10^{10} total cells, at least 9×10^{10} total cells, at least 1×10^{11} total cells of the *Harryflintia acetispora* bacteria. In some embodiments, the pharmaceutical composition comprises no more than 9×10^{11} total cells (e.g., no more than 1×10^{10} total cells, no more than 2×10^{10} total cells, no more than 3×10^{10} total cells, no more than 4×10^{10} total cells, no more than 5×10^{10} total cells, no more than 6×10^{10} total cells, no more than 7×10^{10} total cells, no more than 8×10^{10} total cells, no more than 9×10^{10} total cells, no more than 1×10^{11} total cells, no more than 2×10^{11} total cells, no more than 3×10^{11} total cells, no more than 4×10^{11} total cells, no more than 5×10^{11} total cells, no more than 6×10^{11} total cells, no more than 7×10^{11} total cells, no more than 8×10^{11} total cells) of the *Harryflintia acetispora* bacteria.

[203] In certain aspects, provided herein are pharmaceutical compositions comprising mEVs (such as smEVs and/or pmEVs) useful for the treatment and/or prevention of disease (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease), as well as methods of making and/or identifying such mEVs, and

methods of using such pharmaceutical compositions (e.g., for the treatment and/or prevention of a disease or a health disorder (e.g., adverse health disorders) (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease), either alone or in combination with other therapeutics). In some embodiments, the pharmaceutical compositions comprise both mEVs (such as smEVs and/or pmEVs), and whole bacteria (e.g., live bacteria, killed bacteria, attenuated bacteria). In some embodiments, the pharmaceutical compositions comprise mEVs (such as smEVs and/or pmEVs) in the absence of bacteria. In some embodiments, the pharmaceutical compositions comprise mEVs (such as smEVs and/or pmEVs) and/or bacteria from *Harryflintia acetispora* Strain A.

[204] In certain aspects, provided herein are pharmaceutical compositions comprising *Harryflintia acetispora* bacteria provided and *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs) described herein. In some embodiments, the bacteria is *Harryflintia acetispora* Strain A.

[205] In some embodiments, the bacterial and/or pharmaceutical composition comprises at least 1 *Harryflintia acetispora* bacterium for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , 1×10^8 , 2×10^8 , 3×10^8 , 4×10^8 , 5×10^8 , 6×10^8 , 7×10^8 , 8×10^8 , 9×10^8 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , and/or 1×10^{12} *Harryflintia acetispora* mEV particles.

[206] In some embodiments, the bacterial and/or pharmaceutical composition comprises about 1 *Harryflintia acetispora* bacterium for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , 1×10^8 , 2×10^8 , 3×10^8 , 4×10^8 , 5×10^8 , 6×10^8 , 7×10^8 , 8×10^8 , 9×10^8 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , and/or 1×10^{12} *Harryflintia acetispora* mEV particles.

[207] In some embodiments, the bacterial and/or pharmaceutical composition comprises no more than 1 *Harryflintia acetispora* bacterium for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , 1×10^8 , 2×10^8 , 3×10^8 , 4×10^8 , 5×10^8 , 6×10^8 , 7×10^8 , 8×10^8 , 9×10^8 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 ,

5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , and/or 1×10^{12} *Harryflintia acetispora* mEV particles.

[208] In some embodiments, the bacterial and/or pharmaceutical composition comprises at least 1 *Harryflintia acetispora* mEV particle for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , 4×10^7 , 5×10^7 , 6×10^7 , 7×10^7 , 8×10^7 , 9×10^7 , 1×10^8 , 2×10^8 , 3×10^8 , 4×10^8 , 5×10^8 , 6×10^8 , 7×10^8 , 8×10^8 , 9×10^8 , 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , 9×10^{11} , and/or 1×10^{12} *Harryflintia acetispora* bacteria.

[209] In some embodiments, the bacterial and/or pharmaceutical composition comprises about 1 *Harryflintia acetispora* mEV particle for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 7×10^3 , 8×10^3 , 9×10^3 , 1×10^4 , 2×10^4 , 3×10^4 , 4×10^4 , 5×10^4 , 6×10^4 , 7×10^4 , 8×10^4 , 9×10^4 , 1×10^5 , 2×10^5 ,

3x10⁵, 4x10⁵, 5x10⁵, 6x10⁵, 7x10⁵, 8x10⁵, 9x10⁵, 1x10⁶, 2x10⁶, 3x10⁶, 4x10⁶, 5x10⁶, 6x10⁶, 7x10⁶, 8x10⁶, 9x10⁶, 1x10⁷, 2x10⁷, 3x10⁷, 4x10⁷, 5x10⁷, 6x10⁷, 7x10⁷, 8x10⁷, 9x10⁷, 1x10⁸, 2x10⁸, 3x10⁸, 4x10⁸, 5x10⁸, 6x10⁸, 7x10⁸, 8x10⁸, 9x10⁸, 1x10⁹, 2x10⁹, 3x10⁹, 4x10⁹, 5x10⁹, 6x10⁹, 7x10⁹, 8x10⁹, 9x10⁹, 1x10¹⁰, 2x10¹⁰, 3x10¹⁰, 4x10¹⁰, 5x10¹⁰, 6x10¹⁰, 7x10¹⁰, 8x10¹⁰, 9x10¹⁰, 1x10¹¹, 2x10¹¹, 3x10¹¹, 4x10¹¹, 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, 9x10¹¹, and/or 1x10¹² *Harryflintia acetispora* bacteria.

[210] In some embodiments, the bacterial and/or pharmaceutical composition comprises no more than 1 *Harryflintia acetispora* mEV particle for every 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1x10³, 2x10³, 3x10³, 4x10³, 5x10³, 6x10³, 7x10³, 8x10³, 9x10³, 1x10⁴, 2x10⁴, 3x10⁴, 4x10⁴, 5x10⁴, 6x10⁴, 7x10⁴, 8x10⁴, 9x10⁴, 1x10⁵, 2x10⁵, 3x10⁵, 4x10⁵, 5x10⁵, 6x10⁵, 7x10⁵, 8x10⁵, 9x10⁵, 1x10⁶, 2x10⁶, 3x10⁶, 4x10⁶, 5x10⁶, 6x10⁶, 7x10⁶, 8x10⁶, 9x10⁶, 1x10⁷, 2x10⁷, 3x10⁷, 4x10⁷, 5x10⁷, 6x10⁷, 7x10⁷, 8x10⁷, 9x10⁷, 1x10⁸, 2x10⁸, 3x10⁸, 4x10⁸, 5x10⁸, 6x10⁸, 7x10⁸, 8x10⁸, 9x10⁸, 1x10⁹, 2x10⁹, 3x10⁹, 4x10⁹, 5x10⁹, 6x10⁹, 7x10⁹, 8x10⁹, 9x10⁹, 1x10¹⁰, 2x10¹⁰, 3x10¹⁰, 4x10¹⁰, 5x10¹⁰, 6x10¹⁰, 7x10¹⁰, 8x10¹⁰, 9x10¹⁰, 1x10¹¹, 2x10¹¹, 3x10¹¹, 4x10¹¹, 5x10¹¹, 6x10¹¹, 7x10¹¹, 8x10¹¹, 9x10¹¹, and/or 1x10¹² *Harryflintia acetispora* bacteria.

[211] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[212] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* bacteria.

[213] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of total the particles in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[214] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* bacteria.

[215] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

[216] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are *Harryflintia acetispora* bacteria.

[217] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* mEV protein.

[218] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* bacteria protein.

[219] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* mEV protein.

[220] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* bacteria protein.

[221] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* mEV protein.

[222] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total protein in the pharmaceutical composition is *Harryflintia acetispora* bacteria protein.

[223] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* mEV lipids.

[224] In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* bacteria lipids.

[225] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* mEV lipids.

[226] In some embodiments, no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* bacteria lipids.

[227] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* mEV lipids.

[228] In some embodiments, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* bacteria lipids.

[229] In certain aspects, provided are pharmaceutical compositions for administration to a subject (e.g., human subject). In some embodiments, the pharmaceutical compositions are combined with additional active and/or inactive materials in order to produce a final product, which may be in single dosage unit or in a multi-dose format. In some embodiments, the pharmaceutical composition is combined with an adjuvant such as an immuno-adjuvant (e.g., a STING agonist, a TLR agonist, or a NOD agonist).

[230] In some embodiments, the pharmaceutical composition comprises at least one carbohydrate.

[231] In some embodiments, the pharmaceutical composition comprises at least one lipid. In some embodiments the lipid comprises at least one fatty acid selected from lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), margaric acid (17:0), heptadecenoic acid (17:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), octadecatetraenoic acid (18:4), arachidic acid (20:0), eicosenoic acid (20:1), eicosadienoic acid (20:2), eicosatetraenoic acid (20:4), eicosapentaenoic acid (20:5) (EPA), docosanoic acid (22:0), docosenoic acid (22:1), docosapentaenoic acid (22:5), docosahexaenoic acid (22:6) (DHA), and tetracosanoic acid (24:0).

[232] In some embodiments, the pharmaceutical composition comprises at least one supplemental mineral or mineral source. Examples of minerals include, without limitation: chloride, sodium, calcium, iron, chromium, copper, iodine, zinc, magnesium, manganese, molybdenum, phosphorus, potassium, and selenium. Suitable forms of any of the foregoing minerals include soluble mineral salts, slightly soluble mineral salts, insoluble mineral salts, chelated minerals, mineral complexes, non-reactive minerals such as carbonyl minerals, and reduced minerals, and combinations thereof.

[233] In some embodiments, the pharmaceutical composition comprises at least one supplemental vitamin. The at least one vitamin can be fat-soluble or water soluble vitamins.

Suitable vitamins include but are not limited to vitamin C, vitamin A, vitamin E, vitamin B12, vitamin K, riboflavin, niacin, vitamin D, vitamin B6, folic acid, pyridoxine, thiamine, pantothenic acid, and biotin. Suitable forms of any of the foregoing are salts of the vitamin, derivatives of the vitamin, compounds having the same or similar activity of the vitamin, and metabolites of the vitamin.

[234] In some embodiments, the pharmaceutical composition comprises an excipient. Non-limiting examples of suitable excipients include a buffering agent, a preservative, a stabilizer, a binder, a compaction agent, a lubricant, a dispersion enhancer, a disintegration agent, a flavoring agent, a sweetener, and a coloring agent.

[235] In some embodiments, the excipient is a buffering agent. Non-limiting examples of suitable buffering agents include sodium citrate, magnesium carbonate, magnesium bicarbonate, calcium carbonate, and calcium bicarbonate.

[236] In some embodiments, the excipient comprises a preservative. Non-limiting examples of suitable preservatives include antioxidants, such as alpha-tocopherol and ascorbate, and antimicrobials, such as parabens, chlorobutanol, and phenol.

[237] In some embodiments, the pharmaceutical composition comprises a binder as an excipient. Non-limiting examples of suitable binders include starches, pregelatinized starches, gelatin, polyvinylpyrrolidone, cellulose, methylcellulose, sodium carboxymethylcellulose, ethylcellulose, polyacrylamides, polyvinylloxazolidone, polyvinylalcohols, C₁₂-C₁₈ fatty acid alcohol, polyethylene glycol, polyols, saccharides, oligosaccharides, and combinations thereof.

[238] In some embodiments, the pharmaceutical composition comprises a lubricant as an excipient. Non-limiting examples of suitable lubricants include magnesium stearate, calcium stearate, zinc stearate, hydrogenated vegetable oils, sterotex, polyoxyethylene monostearate, talc, polyethyleneglycol, sodium benzoate, sodium lauryl sulfate, magnesium lauryl sulfate, and light mineral oil.

[239] In some embodiments, the pharmaceutical composition comprises a dispersion enhancer as an excipient. Non-limiting examples of suitable dispersants include starch, alginic acid, polyvinylpyrrolidones, guar gum, kaolin, bentonite, purified wood cellulose, sodium starch glycolate, isoamorphous silicate, and microcrystalline cellulose as high HLB emulsifier surfactants.

[240] In some embodiments, the pharmaceutical composition comprises a disintegrant as an excipient. In some embodiments the disintegrant is a non-effervescent

disintegrant. Non-limiting examples of suitable non-effervescent disintegrants include starches such as corn starch, potato starch, pregelatinized and modified starches thereof, sweeteners, clays, such as bentonite, micro-crystalline cellulose, alginates, sodium starch glycolate, gums such as agar, guar, locust bean, karaya, pectin, and tragacanth. In some embodiments the disintegrant is an effervescent disintegrant. Non-limiting examples of suitable effervescent disintegrants include sodium bicarbonate in combination with citric acid, and sodium bicarbonate in combination with tartaric acid.

[241] In some embodiments, the pharmaceutical composition is a food product (e.g., a food or beverage) such as a health food or beverage, a food or beverage for infants, a food or beverage for pregnant women, athletes, senior citizens or other specified group, a functional food, a beverage, a food or beverage for specified health use, a dietary supplement, a food or beverage for patients, or an animal feed. Specific examples of the foods and beverages include various beverages such as juices, refreshing beverages, tea beverages, drink preparations, jelly beverages, and functional beverages; alcoholic beverages such as beers; carbohydrate-containing foods such as rice food products, noodles, breads, and pastas; paste products such as fish hams, sausages, paste products of seafood; retort pouch products such as curries, food dressed with a thick starchy sauces, and Chinese soups; soups; dairy products such as milk, dairy beverages, ice creams, cheeses, and yogurts; fermented products such as fermented soybean pastes, yogurts, fermented beverages, and pickles; bean products; various confectionery products, including biscuits, cookies, and the like, candies, chewing gums, gummies, cold desserts including jellies, cream caramels, and frozen desserts; instant foods such as instant soups and instant soy-bean soups; microwavable foods; and the like. Further, the examples also include health foods and beverages prepared in the forms of powders, granules, tablets, capsules, liquids, pastes, and jellies.

[242] In some embodiments, the pharmaceutical composition is a food product for animals, including humans. The animals, other than humans, are not particularly limited, and the composition can be used for various livestock, poultry, pets, experimental animals, and the like. Specific examples of the animals include pigs, cattle, horses, sheep, goats, chickens, wild ducks, ostriches, domestic ducks, dogs, cats, rabbits, hamsters, mice, rats, monkeys, and the like, but the animals are not limited thereto.

Dose Forms

[243] A pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, from *Harryflintia acetispora* can be formulated as a solid dose form, e.g., for oral administration. The solid dose form can comprise one or more excipients, e.g., pharmaceutically acceptable excipients. The mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the solid dose form can be isolated mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof. Optionally, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the solid dose form can be lyophilized. Optionally, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the solid dose form are gamma irradiated. The solid dose form can comprise a tablet, a minitab, a capsule, a pill, or a powder; or a combination of these forms (e.g., minitabts comprised in a capsule).

[244] The solid dose form can comprise a tablet (e.g., > 4mm).

[245] The solid dose form can comprise a mini tablet (e.g., 1-4 mm sized minitab, e.g., a 2mm minitab or a 3mm minitab).

[246] The solid dose form can comprise a capsule, e.g., a size 00, size 0, size 1, size 2, size 3, size 4, or size 5 capsule; e.g., a size 0 capsule.

[247] The solid dose form can comprise a coating. The solid dose form can comprise a single layer coating, e.g., enteric coating, e.g., a Eudragit-based coating, e.g., EUDRAGIT L30 D-55, triethylcitrate, and talc. The solid dose form can comprise two layers of coating. For example, an inner coating can comprise, e.g., EUDRAGIT L30 D-55, triethylcitrate, talc, citric acid anhydrous, and sodium hydroxide, and an outer coating can comprise, e.g., EUDRAGIT L30 D-55, triethylcitrate, and talc. EUDRAGIT is the brand name for a diverse range of polymethacrylate-based copolymers. It includes anionic, cationic, and neutral copolymers based on methacrylic acid and methacrylic/acrylic esters or their derivatives. Eudragits are amorphous polymers having glass transition temperatures between 9 to > 150°C. Eudragits are non-biodegradable, nonabsorbable, and nontoxic. Anionic Eudragit L dissolves at pH > 6 and is used for enteric coating, while Eudragit S, soluble at pH > 7 is used for colon targeting. Eudragit RL and RS, having quaternary ammonium groups, are water insoluble, but swellable/permeable polymers which are suitable for the sustained release film coating applications. Cationic Eudragit E, insoluble at pH ≥ 5, can prevent drug release in saliva.

[248] The solid dose form (e.g., a capsule) can comprise a single layer coating, e.g., a non-enteric coating such as HPMC (hydroxyl propyl methyl cellulose) or gelatin.

[249] A pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, from *Harryflintia acetispora* can be formulated as a suspension, e.g., for oral administration or for injection. Administration by injection includes intravenous (IV), intramuscular (IM), intratumoral (IT) and subcutaneous (SC) administration. For a suspension, mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, can be in a buffer, e.g., a pharmaceutically acceptable buffer, e.g., saline or PBS. The suspension can comprise one or more excipients, e.g., pharmaceutically acceptable excipients. The suspension can comprise, e.g., sucrose or glucose. The mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the suspension can be isolated mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof. Optionally, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the suspension can be lyophilized. Optionally, the mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, in the suspension can be gamma irradiated.

Dosage

[250] In some embodiments, the dose of mEVs from *Harryflintia acetispora* bacteria is about 1×10^{11} to about 1×10^{14} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).

[251] As a further example, mEVs from *Harryflintia acetispora* bacteria can be administered at doses e.g., of about 1×10^7 to about 1×10^{15} particles, e.g., as measured by NTA. In some embodiments, the dose of mEVs is about 1×10^5 to about 7×10^{13} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)). In some embodiments, the dose of mEVs from *Harryflintia acetispora* bacteria is about 1×10^{10} to about 7×10^{13} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).

[252] As another example, mEVs from *Harryflintia acetispora* bacteria can be administered at doses e.g., of about 5 mg to about 900 mg total protein, e.g., as measured by Bradford assay. As another example, mEVs from *Harryflintia acetispora* bacteria can be administered at doses e.g., of about 5 mg to about 900 mg total protein, e.g., as measured by BCA assay.

[253] mEVs from *Harryflintia acetispora* can be administered at doses e.g., of about 1×10^7 to about 1×10^{15} particles, e.g., as measured by NTA. In some embodiments, the dose of mEVs is about 1×10^5 to about 7×10^{13} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)). In some embodiments, the dose of mEVs from bacteria is about 1×10^{10} to about 7×10^{13} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)). In some embodiments, the dose of mEVs from bacteria is about 1×10^{11} to about 1×10^{14} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).

[254] As another example, mEVs from *Harryflintia acetispora* can be administered at doses e.g., of about 5 mg to about 900 mg total protein, e.g., as measured by Bradford assay. As another example, mEVs from bacteria can be administered at doses e.g., of about 5 mg to about 900 mg total protein, e.g., as measured by BCA assay.

[255] For oral administration to a human subject, the dose of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, from *Harryflintia acetispora* can be, e.g., about 2×10^6 - about 2×10^{16} particles. The dose can be, e.g., about 1×10^7 - about 1×10^{15} , about 1×10^8 - about 1×10^{14} , about 1×10^9 - about 1×10^{13} , about 1×10^{10} - about 1×10^{14} , or about 1×10^8 - about 1×10^{12} particles. The dose can be, e.g., about 2×10^6 , about 2×10^7 , about 2×10^8 , about 2×10^9 , about 1×10^{10} , about 2×10^{10} , about 2×10^{11} , about 2×10^{12} , about 2×10^{13} , about 2×10^{14} , or about 1×10^{15} particles. The dose can be, e.g., about 2×10^{14} particles. The dose can be, e.g., about 2×10^{12} particles. The dose can be, e.g., about 2×10^{10} particles. The dose can be, e.g., about 1×10^{10} particles. Particle count can be determined, e.g., by NTA. For oral administration to a human subject, the dose of mEVs from *Harryflintia acetispora* can be, e.g., about 2×10^6 - about 2×10^{16} particles. The dose can be, e.g., about 1×10^7 - about 1×10^{15} , about 1×10^8 - about 1×10^{14} , about 1×10^9 - about 1×10^{13} , about 1×10^{10} - about 1×10^{14} , or about 1×10^8 - about 1×10^{12} particles. The dose can be, e.g., about 2×10^6 , about 2×10^7 , about 2×10^8 , about 2×10^9 , about 1×10^{10} , about 2×10^{10} , about 2×10^{11} , about 2×10^{12} , about 2×10^{13} , about 2×10^{14} , or about 1×10^{15} particles. The dose can be, e.g., about 2×10^{14} particles. The dose can be, e.g., about 2×10^{12} particles. The dose can be, e.g., about 2×10^{10} particles. The dose can be, e.g., about 1×10^{10} particles. The dose can be, e.g., about 1×10^{11} to about 1×10^{14} particles. The dose can be, e.g., about 1×10^{11} particles. The dose can be, e.g., about 1×10^{12} particles. The dose can be, e.g., about 1×10^{13} particles. The dose can be, e.g., about 1×10^{14} particles. Particle count can be determined, e.g., by NTA.

[256] For oral administration to a human subject, the dose of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, can be, e.g., based on total protein. The dose can be, e.g., about 5 mg to about 900 mg total protein. The dose can be, e.g., about 20 mg to about 800 mg, about 50 mg to about 700 mg, about 75 mg to about 600 mg, about 100 mg to about 500 mg, about 250 mg to about 750 mg, or about 200 mg to about 500 mg total protein. The dose can be, e.g., about 10 mg, about 25 mg, about 50 mg, about 75 mg, about 100 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, or about 750 mg total protein. Total protein can be determined, e.g., by Bradford assay or BCA assay.

[257] For administration by injection (e.g., intravenous administration) to a human subject, the dose of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, can be, e.g., about 1×10^6 - about 1×10^{16} particles. The dose can be, e.g., about 1×10^7 - about 1×10^{15} , about 1×10^8 - about 1×10^{14} , about 1×10^9 - about 1×10^{13} , about 1×10^{10} - about 1×10^{14} , or about 1×10^8 - about 1×10^{12} particles. The dose can be, e.g., about 2×10^6 , about 2×10^7 , about 2×10^8 , about 2×10^9 , about 1×10^{10} , about 2×10^{10} , about 2×10^{11} , about 2×10^{12} , about 2×10^{13} , about 2×10^{14} , or about 1×10^{15} particles. The dose can be, e.g., about 1×10^{15} particles. The dose can be, e.g., about 2×10^{14} particles. The dose can be, e.g., about 2×10^{13} particles. The dose can be, e.g., about 1×10^{11} to about 1×10^{14} particles. The dose can be, e.g., about 1×10^{11} particles. The dose can be, e.g., about 1×10^{12} particles. The dose can be, e.g., about 1×10^{13} particles. The dose can be, e.g., about 1×10^{14} particles. Particle count can be determined, e.g., by NTA.

[258] For administration by injection (e.g., intravenous administration), the dose of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, can be, e.g., about 5 mg to about 900 mg total protein. The dose can be, e.g., about 20 mg to about 800 mg, about 50 mg to about 700 mg, about 75 mg to about 600 mg, about 100 mg to about 500 mg, about 250 mg to about 750 mg, or about 200 mg to about 500 mg total protein. The dose can be, e.g., about 10 mg, about 25 mg, about 50 mg, about 75 mg, about 100 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, or about 750 mg total protein. The dose can be, e.g., about 700 mg total protein. The dose can be, e.g., about 350 mg total protein. The dose can be, e.g., about 175 mg total protein. Total protein can be determined, e.g., by Bradford assay or BCA assay.

Gamma-irradiation

[259] Powders (e.g., of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof) can be gamma-irradiated at 17.5 kGy radiation unit at ambient temperature.

[260] Frozen biomasses (e.g., of mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof) can be gamma-irradiated at 25 kGy radiation unit in the presence of dry ice.

Additional Therapeutic Agents

[261] In certain aspects, the methods provided herein include the administration to a subject of a pharmaceutical composition described herein either alone or in combination with an additional therapeutic agent. In some embodiments, the additional therapeutic agent is a cancer therapeutic.

[262] In some embodiments, the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, from *Harryflintia acetispora* is administered to the subject before the additional therapeutic agent is administered (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 hours before or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 days before). In some embodiments, the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, is administered to the subject after the additional therapeutic agent is administered (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 hours after or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 days after). In some embodiments, the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, and the additional therapeutic agent are administered to the subject simultaneously or nearly simultaneously (e.g., administrations occur within an hour of each other).

[263] In some embodiments, an antibiotic is administered to the subject before the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, is administered to the subject (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 hours before or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30

days before). In some embodiments, an antibiotic is administered to the subject after pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, is administered to the subject (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 hours before or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 days after). In some embodiments, the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, and the antibiotic are administered to the subject simultaneously or nearly simultaneously (e.g., administrations occur within an hour of each other).

[264] In some embodiments, the additional therapeutic agent is a cancer therapeutic. In some embodiments, the cancer therapeutic is a chemotherapeutic agent. Examples of such chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolmelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin,

marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitioestanol, mepitioestane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitogazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepe; taxoids, e.g., paclitaxel and docetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[265] In some embodiments, the cancer therapeutic is a cancer immunotherapy agent. Immunotherapy refers to a treatment that uses a subject's immune system to treat cancer, e.g., checkpoint inhibitors, cancer vaccines, cytokines, cell therapy, CAR-T cells, and dendritic cell therapy. Non-limiting examples of immunotherapies are checkpoint inhibitors include Nivolumab (BMS, anti-PD-1), Pembrolizumab (Merck, anti-PD-1), Ipilimumab (BMS, anti-CTLA-4), MEDI4736 (AstraZeneca, anti-PD-L1), and MPDL3280A (Roche, anti-PD-L1). Other immunotherapies may be tumor vaccines, such as Gardasil,

Cervarix, BCG, sipulencel-T, Gp100:209-217, AGS-003, DCVax-L, Algenpantucel-L, Tergenpantucel-L, TG4010, ProstAtak, Prostavac-V/R-TRICOM, Rindopepimul, E75 peptide acetate, IMA901, POL-103A, Belagenpumatucel-L, GSK1572932A, MDX-1279, GV1001, and Tecemotide. The immunotherapy agent may be administered via injection (e.g., intravenously, intratumorally, subcutaneously, or into lymph nodes), but may also be administered orally, topically, or via aerosol. Immunotherapies may comprise adjuvants such as cytokines.

[266] In some embodiments, the immunotherapy agent is an immune checkpoint inhibitor. Immune checkpoint inhibition broadly refers to inhibiting the checkpoints that cancer cells can produce to prevent or downregulate an immune response. Examples of immune checkpoint proteins include, but are not limited to, CTLA4, PD-1, PD-L1, PD-L2, A2AR, B7-H3, B7-H4, BTLA, KIR, LAG3, TIM-3 or VISTA. Immune checkpoint inhibitors can be antibodies or antigen binding fragments thereof that bind to and inhibit an immune checkpoint protein. Examples of immune checkpoint inhibitors include, but are not limited to, nivolumab, pembrolizumab, pidilizumab, AMP-224, AMP-514, STI-A1110, TSR-042, RG-7446, BMS-936559, MEDI-4736, MSB-0010718C (avelumab), AUR-012 and STI-A1010. In some embodiments, the immune checkpoint inhibitor is a CTLA-4 inhibitor. In some embodiments, the immune checkpoint inhibitor is a PD-1 inhibitor. In some embodiments, the immune checkpoint inhibitor is a PD-L1 inhibitor. In some embodiments, the immune checkpoint inhibitor is an antibody.

[267] In some embodiments, the methods provided herein include the administration of a pharmaceutical composition described herein in combination with one or more additional therapeutic agents. In some embodiments, the methods disclosed herein include the administration of two immunotherapy agents (e.g., immune checkpoint inhibitor). For example, the methods provided herein include the administration of a pharmaceutical composition described herein in combination with a PD-1 inhibitor (such as pemrolizumab or nivolumab or pidilizumab) or a CLTA-4 inhibitor (such as ipilimumab) or a PD-L1 inhibitor (such as avelumab).

[268] In some embodiments, the immunotherapy agent is an antibody or antigen binding fragment thereof that, for example, binds to a cancer-associated antigen. Examples of cancer-associated antigens include, but are not limited to, adipophilin, AIM-2, ALDH1A1, alpha-actinin-4, alpha-fetoprotein ("AFP"), ARTC1, B-RAF, BAGE-1, BCLX (L), BCR-ABL fusion protein b3a2, beta-catenin, BING-4, CA-125, CALCA,

carcinoembryonic antigen ("CEA"), CASP-5, CASP-8, CD274, CD45, Cdc27, CDK12, CDK4, CDKN2A, CEA, CLPP, COA-1, CPSE, CSNK1A1, CTAG1, CTAG2, cyclin D1, Cyclin-A1, dek-can fusion protein, DKK1, EFTUD2, Elongation factor 2, ENAH (hMena), Ep-CAM, EpCAM, EphA3, epithelial tumor antigen ("ETA"), ETV6-AML1 fusion protein, EZH2, FGF5, FLT3-ITD, FN1, G250/MN/CAIX, GAGE-1,2,8, GAGE-3,4,5,6,7, GAS7, glypican-3, GnTV, gp100/Pmel17, GPNMB, HAUS3, Hepsin, HER-2/neu, HERV-K-MEL, HLA-A11, HLA-A2, HLA-DOB, hsp70-2, IDO1, IGF2B3, IL13Ralpha2, Intestinal carboxyl esterase, K-ras, Kallikrein 4, KIF20A, KK-LC-1, KKLC1, KM-HN-1, KMHN1 also known as CCDC110, LAGE-1, LDLR-fucosyltransferaseAS fusion protein, Lengsin, M-CSF, MAGE-A1, MAGE-A10, MAGE-A12, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-C1, MAGE-C2, malic enzyme, mammaglobin-A, MART2, MATN, MC1R, MCSP, mdm-2, ME1, Melan-A/MART-1, Meloe, Midkine, MMP-2, MMP-7, MUC1, MUC5AC, mucin, MUM-1, MUM-2, MUM-3, Myosin, Myosin class I, N-rav, NA88-A, neo-PAP, NFYC, NY-BR-1, NY-ESO-1/LAGE-2, OA1, OGT, OS-9, P polypeptide, p53, PAP, PAX5, PBF, pml-RARalpha fusion protein, polymorphic epithelial mucin ("PEM"), PPP1R3B, PRAME, PRDX5, PSA, PSMA, PTPRK, RAB38/NY-MEL-1, RAGE-1, RBAF600, RGS5, RhoC, RNF43, RU2AS, SAGE, secernin 1, SIRT2, SNRPD1, SOX10, Sp17, SPA17, SSX-2, SSX-4, STEAP1, survivin, SYT-SSX1 or -SSX2 fusion protein, TAG-1, TAG-2, Telomerase, TGF-betaRII, TPBG, TRAG-3, Triosephosphate isomerase, TRP-1/gp75, TRP-2, TRP2-INT2, tyrosinase, tyrosinase ("TYR"), VEGF, WT1, XAGE-1b/GAGED2a. In some embodiments, the antigen is a neo-antigen.

[269] In some embodiments, the immunotherapy agent is a cancer vaccine and/or a component of a cancer vaccine (e.g., an antigenic peptide and/or protein). The cancer vaccine can be a protein vaccine, a nucleic acid vaccine or a combination thereof. For example, in some embodiments, the cancer vaccine comprises a polypeptide comprising an epitope of a cancer-associated antigen. In some embodiments, the cancer vaccine comprises a nucleic acid (e.g., DNA or RNA, such as mRNA) that encodes an epitope of a cancer-associated antigen. Examples of cancer-associated antigens include, but are not limited to, adipophilin, AIM-2, ALDH1A1, alpha-actinin-4, alpha-fetoprotein ("AFP"), ARTC1, B-RAF, BAGE-1, BCLX (L), BCR-ABL fusion protein b3a2, beta-catenin, BING-4, CA-125, CALCA, carcinoembryonic antigen ("CEA"), CASP-5, CASP-8, CD274, CD45, Cdc27, CDK12, CDK4, CDKN2A, CEA, CLPP, COA-1, CPSE, CSNK1A1, CTAG1, CTAG2, cyclin D1, Cyclin-A1, dek-can fusion protein, DKK1, EFTUD2, Elongation factor 2, ENAH

(hMena), Ep-CAM, EpCAM, EphA3, epithelial tumor antigen ("ETA"), ETV6-AML1 fusion protein, EZH2, FGF5, FLT3-ITD, FN1, G250/MN/CAIX, GAGE-1,2,8, GAGE-3,4,5,6,7, GAS7, glypican-3, GnTV, gp100/Pmel17, GPNMB, HAUS3, Hepsin, HER-2/neu, HERV-K-MEL, HLA-A11, HLA-A2, HLA-DOB, hsp70-2, IDO1, IGF2B3, IL13Ralpha2, Intestinal carboxyl esterase, K-ras, Kallikrein 4, KIF20A, KK-LC-1, KKLC1, KM-HN-1, KMHN1 also known as CCDC110, LAGE-1, LDLR-fucosyltransferaseAS fusion protein, Lengsin, M-CSF, MAGE-A1, MAGE-A10, MAGE-A12, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-C1, MAGE-C2, malic enzyme, mammaglobin-A, MART2, MATN, MC1R, MCSP, mdm-2, ME1, Melan-A/MART-1, Meloc, Midkine, MMP-2, MMP-7, MUC1, MUC5AC, mucin, MUM-1, MUM-2, MUM-3, Myosin, Myosin class I, N-ras, NA88-A, neo-PAP, NFYC, NY-BR-1, NY-ESO-1/LAGE-2, OA1, OGT, OS-9, P polypeptide, p53, PAP, PAX5, PBF, pml-RARalpha fusion protein, polymorphic epithelial mucin ("PEM"), PPP1R3B, PRAME, PRDX5, PSA, PSMA, PTPRK, RAB38/NY-MEL-1, RAGE-1, RBAF600, RGS5, RhoC, RNF43, RU2AS, SAGE, secernin 1, SIRT2, SNRPD1, SOX10, Sp17, SPA17, SSX-2, SSX-4, STEAP1, survivin, SYT-SSX1 or -SSX2 fusion protein, TAG-1, TAG-2, Telomerase, TGF-betaRII, TPBG, TRAG-3, Triosephosphate isomerase, TRP-1/gp75, TRP-2, TRP2-INT2, tyrosinase, tyrosinase ("TYR"), VEGF, WT1, XAGE-1b/GAGED2a. In some embodiments, the antigen is a neo-antigen. In some embodiments, the cancer vaccine is administered with an adjuvant. Examples of adjuvants include, but are not limited to, an immune modulatory protein, Adjuvant 65, α -GalCer, aluminum phosphate, aluminum hydroxide, calcium phosphate, β -Glucan Peptide, CpG ODN DNA, GPI-0100, lipid A, lipopolysaccharide, Lipovant, Montanide, N-acetyl-muramyl-L-alanyl-D-isoglutamine, Pam3CSK4, quil A, cholera toxin (CT) and heat-labile toxin from enterotoxigenic *Escherichia coli* (LT) including derivatives of these (CTB, mmCT, CTA1-DD, LTB, LTK63, LTR72, dmLT) and trehalose dimycolate. [270]

In some embodiments, the immunotherapy agent is an immune modulating protein to the subject. In some embodiments, the immune modulatory protein is a cytokine or chemokine. Examples of immune modulating proteins include, but are not limited to, B lymphocyte chemoattractant ("BLC"), C-C motif chemokine 11 ("Eotaxin-1"), Eosinophil chemotactic protein 2 ("Eotaxin-2"), Granulocyte colony-stimulating factor ("G-CSF"), Granulocyte macrophage colony-stimulating factor ("GM-CSF"), 1-309, Intercellular Adhesion Molecule 1 ("ICAM-1"), Interferon alpha ("IFN-alpha"), Interferon beta ("IFN-beta") Interferon gamma ("IFN-gamma"), Interleukin-1 alpha ("IL-1 alpha"), Interleukin-1

beta ("IL-1 beta"), Interleukin 1 receptor antagonist ("IL-1 ra"), Interleukin-2 ("IL-2"), Interleukin-4 ("IL-4"), Interleukin-5 ("IL-5"), Interleukin-6 ("IL-6"), Interleukin-6 soluble receptor ("IL-6 sR"), Interleukin-7 ("IL-7"), Interleukin-8 ("IL-8"), Interleukin-10 ("IL-10"), Interleukin-11 ("IL-11"), Subunit beta of Interleukin-12 ("IL-12 p40" or "IL-12 p70"), Interleukin-13 ("IL-13"), Interleukin-15 ("IL-15"), Interleukin-16 ("IL-16"), Interleukin-17A-F ("IL-17A-F"), Interleukin-18 ("IL-18"), Interleukin-21 ("IL-21"), Interleukin-22 ("IL-22"), Interleukin-23 ("IL-23"), Interleukin-33 ("IL-33"), Chemokine (C-C motif) Ligand 2 ("MCP-1"), Macrophage colony-stimulating factor ("M-CSF"), Monokine induced by gamma interferon ("MIG"), Chemokine (C-C motif) ligand 2 ("MIP-1 alpha"), Chemokine (C-C motif) ligand 4 ("MIP-1 beta"), Macrophage inflammatory protein-1-delta ("MIP-1 delta"), Platelet-derived growth factor subunit B ("PDGF-BB"), Chemokine (C-C motif) ligand 5, Regulated on Activation, Normal T cell Expressed and Secreted ("RANTES"), TIMP metalloproteinase inhibitor 1 ("TIMP-1"), TIMP metalloproteinase inhibitor 2 ("TIMP-2"), Tumor necrosis factor, lymphotoxin-alpha ("TNF alpha"), Tumor necrosis factor, lymphotoxin-beta ("TNF beta"), Soluble TNF receptor type 1 ("sTNFR1"), sTNFR1AR, Brain-derived neurotrophic factor ("BDNF"), Basic fibroblast growth factor ("bFGF"), Bone morphogenetic protein 4 ("BMP-4"), Bone morphogenetic protein 5 ("BMP-5"), Bone morphogenetic protein 7 ("BMP-7"), Nerve growth factor ("b-NGF"), Epidermal growth factor ("EGF"), Epidermal growth factor receptor ("EGFR"), Endocrine-gland-derived vascular endothelial growth factor ("EG-VEGF"), Fibroblast growth factor 4 ("FGF-4"), Keratinocyte growth factor ("FGF-7"), Growth differentiation factor 15 ("GDF-15"), Glial cell-derived neurotrophic factor ("GDNF"), Growth Hormone, Heparin-binding EGF-like growth factor ("HB-EGF"), Hepatocyte growth factor ("HGF"), Insulin-like growth factor binding protein 1 ("IGFBP-1"), Insulin-like growth factor binding protein 2 ("IGFBP-2"), Insulin-like growth factor binding protein 3 ("IGFBP-3"), Insulin-like growth factor binding protein 4 ("IGFBP-4"), Insulin-like growth factor binding protein 6 ("IGFBP-6"), Insulin-like growth factor 1 ("IGF-1"), Insulin, Macrophage colony-stimulating factor ("M-CSF R"), Nerve growth factor receptor ("NGF R"), Neurotrophin-3 ("NT-3"), Neurotrophin-4 ("NT-4"), Osteoclastogenesis inhibitory factor ("Osteoprotegerin"), Platelet-derived growth factor receptors ("PDGF-AA"), Phosphatidylinositol-glycan biosynthesis ("PIGF"), Skp, Cullin, F-box containing complex ("SCF"), Stem cell factor receptor ("SCF R"), Transforming growth factor alpha ("TGFalpha"), Transforming growth factor beta-1 ("TGF beta 1"), Transforming growth factor beta-3 ("TGF beta 3"), Vascular endothelial growth factor ("VEGF"),

Vascular endothelial growth factor receptor 2 ("VEGFR2"), Vascular endothelial growth factor receptor 3 ("VEGFR3"), VEGF-D 6Ckine, Tyrosine-protein kinase receptor UFO ("Axl"), Betacellulin ("BTC"), Mucosae-associated epithelial chemokine ("CCL28"), Chemokine (C-C motif) ligand 27 ("CTACK"), Chemokine (C-X-C motif) ligand 16 ("CXCL16"), C-X-C motif chemokine 5 ("ENA-78"), Chemokine (C-C motif) ligand 26 ("Eotaxin-3"), Granulocyte chemotactic protein 2 ("GCP-2"), GRO, Chemokine (C-C motif) ligand 14 ("HCC-1"), Chemokine (C-C motif) ligand 16 ("HCC-4"), Interleukin-9 ("IL-9"), Interleukin-17 F ("IL-17F"), Interleukin-18-binding protein ("IL-18 BP α "), Interleukin-28 A ("IL-28A"), Interleukin 29 ("IL-29"), Interleukin 31 ("IL-31"), C-X-C motif chemokine 10 ("IP-10"), Chemokine receptor CXCR3 ("I-TAC"), Leukemia inhibitory factor ("LIF"), Light, Chemokine (C motif) ligand ("Lymphotactin"), Monocyte chemoattractant protein 2 ("MCP-2"), Monocyte chemoattractant protein 3 ("MCP-3"), Monocyte chemoattractant protein 4 ("MCP-4"), Macrophage-derived chemokine ("MDC"), Macrophage migration inhibitory factor ("MIF"), Chemokine (C-C motif) ligand 20 ("MIP-3 α "), C-C motif chemokine 19 ("MIP-3 β "), Chemokine (C-C motif) ligand 23 ("MPIF-1"), Macrophage stimulating protein α chain ("MSP α "), Nucleosome assembly protein 1-like 4 ("NAP-2"), Secreted phosphoprotein 1 ("Osteopontin"), Pulmonary and activation-regulated cytokine ("PARC"), Platelet factor 4 ("PF4"), Stroma cell-derived factor-1 α ("SDF-1 α "), Chemokine (C-C motif) ligand 17 ("TARC"), Thymus-expressed chemokine ("TECK"), Thymic stromal lymphopoietin ("TSLP 4-IBB"), CD 166 antigen ("ALCAM"), Cluster of Differentiation 80 ("B7-1"), Tumor necrosis factor receptor superfamily member 17 ("BCMA"), Cluster of Differentiation 14 ("CD14"), Cluster of Differentiation 30 ("CD30"), Cluster of Differentiation 40 ("CD40 Ligand"), Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) ("CEACAM-1"), Death Receptor 6 ("DR6"), Deoxythymidine kinase ("Dtk"), Type 1 membrane glycoprotein ("Endoglin"), Receptor tyrosine-protein kinase erbB-3 ("ErbB3"), Endothelial-leukocyte adhesion molecule 1 ("E-Selectin"), Apoptosis antigen 1 ("Fas"), Fms-like tyrosine kinase 3 ("Flt-3L"), Tumor necrosis factor receptor superfamily member 1 ("GITR"), Tumor necrosis factor receptor superfamily member 14 ("HVEM"), Intercellular adhesion molecule 3 ("ICAM-3"), IL-1 R4, IL-1 RI, IL-10 R β , IL-17R, IL-2R γ , IL-21R, Lysosome membrane protein 2 ("LIMPII"), Neutrophil gelatinase-associated lipocalin ("Lipocalin-2"), CD62L ("L-Selectin"), Lymphatic endothelium ("LYVE-1"), MHC class I polypeptide-related sequence A ("MICA"), MHC class I polypeptide-related sequence B ("MICB"), NRG1- β tal, Beta-

type platelet-derived growth factor receptor ("PDGF Rbeta"), Platelet endothelial cell adhesion molecule ("PECAM-1"), RAGE, Hepatitis A virus cellular receptor 1 ("TIM-1"), Tumor necrosis factor receptor superfamily member 10C ("TRAIL R3"), Trappin protein transglutaminase binding domain ("Trappin-2"), Urokinase receptor ("uPAR"), Vascular cell adhesion protein 1 ("VCAM-1"), XEDARActivin A, Agouti-related protein ("AgRP"), Ribonuclease 5 ("Angiogenin"), Angiopoietin 1, Angiostatin, Cathepsin S, CD40, Cryptic family protein 1B ("Cripto-1"), DAN, Dickkopf-related protein 1 ("DKK-1"), E-Cadherin, Epithelial cell adhesion molecule ("EpCAM"), Fas Ligand (FasL or CD95L), Fcg RIIB/C, Follistatin, Galectin-7, Intercellular adhesion molecule 2 ("ICAM-2"), IL-13 R1, IL-13R2, IL-17B, IL-2 Ra, IL-2 Rb, IL-23, LAP, Neuronal cell adhesion molecule ("NrcAM"), Plasminogen activator inhibitor- 1 ("PAI-1"), Platelet derived growth factor receptors ("PDGF-AB"), Resistin, stromal cell-derived factor 1 ("SDF-1 beta"), sgpl30, Secreted frizzled-related protein 2 ("ShhN"), Sialic acid-binding immunoglobulin-type lectins ("Siglec-5"), ST2, Transforming growth factor-beta 2 ("TGF beta 2"), Tie-2, Thrombopoietin ("TPO"), Tumor necrosis factor receptor superfamily member 10D ("TRAIL R4"), Triggering receptor expressed on myeloid cells 1 ("TREM-1"), Vascular endothelial growth factor C ("VEGF-C"), VEGFR1Adiponectin, Adipsin ("AND"), Alpha-fetoprotein ("AFP"), Angiopoietin-like 4 ("ANGPTL4"), Beta-2-microglobulin ("B2M"), Basal cell adhesion molecule ("BCAM"), Carbohydrate antigen 125 ("CA125"), Cancer Antigen 15-3 ("CA15-3"), Carcinoembryonic antigen ("CEA"), cAMP receptor protein ("CRP"), Human Epidermal Growth Factor Receptor 2 ("ErbB2"), Follistatin, Follicle-stimulating hormone ("FSH"), Chemokine (C-X-C motif) ligand 1 ("GRO alpha"), human chorionic gonadotropin ("beta HCG"), Insulin-like growth factor 1 receptor ("IGF-1 sR"), IL-1 sRII, IL-3, IL-18 Rb, IL-21, Leptin, Matrix metalloproteinase-1 ("MMP-1"), Matrix metalloproteinase-2 ("MMP-2"), Matrix metalloproteinase-3 ("MMP-3"), Matrix metalloproteinase-8 ("MMP-8"), Matrix metalloproteinase-9 ("MMP-9"), Matrix metalloproteinase-10 ("MMP-10"), Matrix metalloproteinase-13 ("MMP-13"), Neural Cell Adhesion Molecule ("NCAM-1"), Entactin ("Nidogen-1"), Neuron specific enolase ("NSE"), Oncostatin M ("OSM"), Procalcitonin, Prolactin, Prostate specific antigen ("PSA"), Sialic acid-binding Ig-like lectin 9 ("Siglec-9"), ADAM 17 endopeptidase ("TACE"), Thyroglobulin, Metalloproteinase inhibitor 4 ("TIMP-4"), TSH2B4, Disintegrin and metalloproteinase domain-containing protein 9 ("ADAM-9"), Angiopoietin 2, Tumor necrosis factor ligand superfamily member 13/ Acidic leucine-rich nuclear phosphoprotein

32 family member B ("APRIL"), Bone morphogenetic protein 2 ("BMP-2"), Bone morphogenetic protein 9 ("BMP-9"), Complement component 5a ("C5a"), Cathepsin L, CD200, CD97, Chemerin, Tumor necrosis factor receptor superfamily member 6B ("DcR3"), Fatty acid-binding protein 2 ("FABP2"), Fibroblast activation protein, alpha ("FAP"), Fibroblast growth factor 19 ("FGF-19"), Galectin-3, Hepatocyte growth factor receptor ("HGF R"), IFN-gamma/beta R2, Insulin-like growth factor 2 ("IGF-2"), Insulin-like growth factor 2 receptor ("IGF-2 R"), Interleukin-1 receptor 6 ("IL-1R6"), Interleukin 24 ("IL-24"), Interleukin 33 ("IL-33"), Kallikrein 14, Asparaginyl endopeptidase ("Legumain"), Oxidized low-density lipoprotein receptor 1 ("LOX-1"), Mannose-binding lectin ("MBL"), Neprilysin ("NEP"), Notch homolog 1, translocation-associated (*Drosophila*) ("Notch-1"), Nephroblastoma overexpressed ("NOV"), Osteoactivin, Programmed cell death protein 1 ("PD-1"), N-acetylmuramoyl-L-alanine amidase ("PGRP-5"), Serpin A4, Secreted frizzled related protein 3 ("sFRP-3"), Thrombomodulin, Toll-like receptor 2 ("TLR2"), Tumor necrosis factor receptor superfamily member 10A ("TRAIL R1"), Transferrin ("TRF"), WIF-1/ACE-2, Albumin, AMICA, Angiopoietin 4, B-cell activating factor ("BAFF"), Carbohydrate antigen 19-9 ("CA19-9"), CD 163, Clusterin, CRT AM, Chemokine (C-X-C motif) ligand 14 ("CXCL14"), Cystatin C, Decorin ("DCN"), Dickkopf-related protein 3 ("Dkk-3"), Delta-like protein 1 ("DLL1"), Fetuin A, Heparin-binding growth factor 1 ("aFGF"), Folate receptor alpha ("FOLR1"), Furin, GPCR-associated sorting protein 1 ("GASP-1"), GPCR-associated sorting protein 2 ("GASP-2"), Granulocyte colony-stimulating factor receptor ("GCSF R"), Serine protease hepsin ("HAI-2"), Interleukin-17B Receptor ("IL-17B R"), Interleukin 27 ("IL-27"), Lymphocyte-activation gene 3 ("LAG-3"), Apolipoprotein A-V ("LDL R"), Pepsinogen I, Retinol binding protein 4 ("RBP4"), SOST, Heparan sulfate proteoglycan ("Syndecan-1"), Tumor necrosis factor receptor superfamily member 13B ("TACI"), Tissue factor pathway inhibitor ("TFPI"), TSP-1, Tumor necrosis factor receptor superfamily, member 10b ("TRAIL R2"), TRANCE, Troponin I, Urokinase Plasminogen Activator ("uPA"), Cadherin 5, type 2 or VE-cadherin (vascular endothelial) also known as CD144 ("VE-Cadherin"), WNT1-inducible-signaling pathway protein 1 ("WISP-1"), and Receptor Activator of Nuclear Factor κ B ("RANK").

[271] In some embodiments, the cancer therapeutic is an anti-cancer compound. Exemplary anti-cancer compounds include, but are not limited to, Alemtuzumab (Campath®), Alitretinoin (Panretin®), Anastrozole (Arimidex®), Bevacizumab (Avastin®),

Bexarotene (Targretin®), Bortezomib (Velcade®), Bosutinib (Bosulif®), Brentuximab vedotin (Adcetris®), Cabozantinib (Cometriq™), Carfilzomib (Kyprolis™), Cetuximab (Erbix®), Crizotinib (Xalkori®), Dasatinib (Sprycel®), Denileukin diftitox (Ontak®), Erlotinib hydrochloride (Tarceva®), Everolimus (Afinitor®), Exemestane (Aromasin®), Fulvestrant (Faslodex®), Gefitinib (Iressa®), Ibritumomab tiuxetan (Zevalin®), Imatinib mesylate (Gleevec®), Ipilimumab (Yervoy™), Lapatinib ditosylate (Tykerb®), Letrozole (Femara®), Nilotinib (Tasigna®), Ofatumumab (Arzerra®), Panitumumab (Vectibix®), Pazopanib hydrochloride (Votrient®), Pertuzumab (Perjeta™), Pralatrexate (Folotyn®), Regorafenib (Stivarga®), Rituximab (Rituxan®), Romidepsin (Istodax®), Sorafenib tosylate (Nexavar®), Sunitinib malate (Sutent®), Tamoxifen, Temsirolimus (Torisel®), Toremifene (Fareston®), Tositumomab and ¹³¹I-tositumomab (Bexxar®), Trastuzumab (Herceptin®), Tretinoin (Vesanoid®), Vandetanib (Caprelsa®), Vemurafenib (Zelboraf®), Vorinostat (Zolinza®), and Ziv-aflibercept (Zaltrap®).

[272] Exemplary anti-cancer compounds that modify the function of proteins that regulate gene expression and other cellular functions (e.g., HDAC inhibitors, retinoid receptor ligands) are Vorinostat (Zolinza®), Bexarotene (Targretin®) and Romidepsin (Istodax®), Alitretinoin (Panretin®), and Tretinoin (Vesanoid®).

[273] Exemplary anti-cancer compounds that induce apoptosis (e.g., proteasome inhibitors, antifolates) are Bortezomib (Velcade®), Carfilzomib (Kyprolis™), and Pralatrexate (Folotyn®).

[274] Exemplary anti-cancer compounds that increase anti-tumor immune response (e.g., anti CD20, anti CD52; anti-cytotoxic T-lymphocyte-associated antigen-4) are Rituximab (Rituxan®), Alemtuzumab (Campath®), Ofatumumab (Arzerra®), and Ipilimumab (Yervoy™).

[275] Exemplary anti-cancer compounds that deliver toxic agents to cancer cells (e.g., anti-CD20-radionuclide fusions; IL-2-diphtheria toxin fusions; anti-CD30-monomethylauristatin E (MMAE)-fusions) are Tositumomab and ¹³¹I-tositumomab (Bexxar®) and Ibritumomab tiuxetan (Zevalin®), Denileukin diftitox (Ontak®), and Brentuximab vedotin (Adcetris®).

[276] Other exemplary anti-cancer compounds are small molecule inhibitors and conjugates thereof of, e.g., Janus kinase, ALK, Bcl-2, PARP, PI3K, VEGF receptor, Braf, MEK, CDK, and HSP90.

[277] Exemplary platinum-based anti-cancer compounds include, for example, cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, Nedaplatin, Triplatin, and Lipoplatin. Other metal-based drugs suitable for treatment include, but are not limited to ruthenium-based compounds, ferrocene derivatives, titanium-based compounds, and gallium-based compounds.

[278] In some embodiments, the cancer therapeutic is a radioactive moiety that comprises a radionuclide. Exemplary radionuclides include, but are not limited to Cr-51, Cs-131, Ce-134, Se-75, Ru-97, I-125, Eu-149, Os-189m, Sb-119, I-123, Ho-161, Sb-117, Ce-139, In-111, Rh-103m, Ga-67, Tl-201, Pd-103, Au-195, Hg-197, Sr-87m, Pt-191, P-33, Er-169, Ru-103, Yb-169, Au-199, Sn-121, Tm-167, Yb-175, In-113m, Sn-113, Lu-177, Rh-105, Sn-117m, Cu-67, Sc-47, Pt-195m, Ce-141, I-131, Tb-161, As-77, Pt-197, Sm-153, Gd-159, Tm-173, Pr-143, Au-198, Tm-170, Re-186, Ag-111, Pd-109, Ga-73, Dy-165, Pm-149, Sn-123, Sr-89, Ho-166, P-32, Re-188, Pr-142, Ir-194, In-114m/In-114, and Y-90.

[279] In some embodiments, the cancer therapeutic is an antibiotic. For example, if the presence of a cancer-associated bacteria and/or a cancer-associated microbiome profile is detected according to the methods provided herein, antibiotics can be administered to eliminate the cancer-associated bacteria from the subject. "Antibiotics" broadly refers to compounds capable of inhibiting or preventing a bacterial infection. Antibiotics can be classified in a number of ways, including their use for specific infections, their mechanism of action, their bioavailability, or their spectrum of target microbe (e.g., Gram-negative vs. Gram-positive bacteria, aerobic vs. anaerobic bacteria, etc.) and these may be used to kill specific bacteria in specific areas of the host ("niches") (Leekha, et al 2011. General Principles of Antimicrobial Therapy. Mayo Clin Proc. 86(2): 156-167). In certain embodiments, antibiotics can be used to selectively target bacteria of a specific niche. In some embodiments, antibiotics known to treat a particular infection that includes a cancer niche may be used to target cancer-associated microbes, including cancer-associated bacteria in that niche. In other embodiments, antibiotics are administered after the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof. In some embodiments, antibiotics are administered before pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof.

[280] In some aspects, antibiotics can be selected based on their bactericidal or bacteriostatic properties. Bactericidal antibiotics include mechanisms of action that disrupt

the cell wall (e.g., β -lactams), the cell membrane (e.g., daptomycin), or bacterial DNA (e.g., fluoroquinolones). Bacteriostatic agents inhibit bacterial replication and include sulfonamides, tetracyclines, and macrolides, and act by inhibiting protein synthesis. Furthermore, while some drugs can be bactericidal in certain organisms and bacteriostatic in others, knowing the target organism allows one skilled in the art to select an antibiotic with the appropriate properties. In certain treatment conditions, bacteriostatic antibiotics inhibit the activity of bactericidal antibiotics. Thus, in certain embodiments, bactericidal and bacteriostatic antibiotics are not combined.

[281] Antibiotics include, but are not limited to aminoglycosides, ansamycins, carbacephems, carbapenems, cephalosporins, glycopeptides, lincosamides, lipopeptides, macrolides, monobactams, nitrofurans, oxazolidinones, penicillins, polypeptide antibiotics, quinolones, fluoroquinolone, sulfonamides, tetracyclines, and anti-mycobacterial compounds, and combinations thereof.

[282] Aminoglycosides include, but are not limited to Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Tobramycin, Paromomycin, and Spectinomycin. Aminoglycosides are effective, e.g., against Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, and *Francisella tularensis*, and against certain aerobic bacteria but less effective against obligate/facultative anaerobes. Aminoglycosides are believed to bind to the bacterial 30S or 50S ribosomal subunit thereby inhibiting bacterial protein synthesis.

[283] Ansamycins include, but are not limited to, Geldanamycin, Herbimycin, Rifamycin, and Streptovaricin. Geldanamycin and Herbimycin are believed to inhibit or alter the function of Heat Shock Protein 90.

[284] Carbacephems include, but are not limited to, Loracarbef. Carbacephems are believed to inhibit bacterial cell wall synthesis.

[285] Carbapenems include, but are not limited to, Ertapenem, Doripenem, Imipenem/Cilastatin, and Meropenem. Carbapenems are bactericidal for both Gram-positive and Gram-negative bacteria as broad-spectrum antibiotics. Carbapenems are believed to inhibit bacterial cell wall synthesis.

[286] Cephalosporins include, but are not limited to, Cefadroxil, Cefazolin, Cefalotin, Cefalothin, Cefalexin, Cefaclor, Cefamandole, Cefoxitin, Cefprozil, Cefuroxime, Cefixime, Cefdinir, Cefditoren, Cefoperazone, Cefotaxime, Cefpodoxime, Ceftazidime, Ceftibuten, Ceftizoxime, Ceftriaxone, Cefepime, Ceftaroline fosamil, and Ceftobiprole.

Selected Cephalosporins are effective, e.g., against Gram-negative bacteria and against Gram-positive bacteria, including *Pseudomonas*, certain Cephalosporins are effective against methicillin-resistant *Staphylococcus aureus* (MRSA). Cephalosporins are believed to inhibit bacterial cell wall synthesis by disrupting synthesis of the peptidoglycan layer of bacterial cell walls.

[287] Glycopeptides include, but are not limited to, Teicoplanin, Vancomycin, and Telavancin. Glycopeptides are effective, e.g., against aerobic and anaerobic Gram-positive bacteria including MRSA and *Clostridium difficile*. Glycopeptides are believed to inhibit bacterial cell wall synthesis by disrupting synthesis of the peptidoglycan layer of bacterial cell walls.

[288] Lincosamides include, but are not limited to, Clindamycin and Lincomycin. Lincosamides are effective, e.g., against anaerobic bacteria, as well as *Staphylococcus*, and *Streptococcus*. Lincosamides are believed to bind to the bacterial 50S ribosomal subunit thereby inhibiting bacterial protein synthesis.

[289] Lipopeptides include, but are not limited to, Daptomycin. Lipopeptides are effective, e.g., against Gram-positive bacteria. Lipopeptides are believed to bind to the bacterial membrane and cause rapid depolarization.

[290] Macrolides include, but are not limited to, Azithromycin, Clarithromycin, Dirithromycin, Erythromycin, Roxithromycin, Troleandomycin, Telithromycin, and Spiramycin. Macrolides are effective, e.g., against *Streptococcus* and *Mycoplasma*. Macrolides are believed to bind to the bacterial or 50S ribosomal subunit, thereby inhibiting bacterial protein synthesis.

[291] Monobactams include, but are not limited to, Aztreonam. Monobactams are effective, e.g., against Gram-negative bacteria. Monobactams are believed to inhibit bacterial cell wall synthesis by disrupting synthesis of the peptidoglycan layer of bacterial cell walls.

[292] Nitrofurans include, but are not limited to, Furazolidone and Nitrofurantoin.

[293] Oxazolidinones include, but are not limited to, Linezolid, Posizolid, Radezolid, and Torezolid. Oxazolidinones are believed to be protein synthesis inhibitors.

[294] Penicillins include, but are not limited to, Amoxicillin, Ampicillin, Azlocillin, Carbenicillin, Cloxacillin, Dicloxacillin, Flucloxacillin, Mezlocillin, Methicillin, Nafcillin, Oxacillin, Penicillin G, Penicillin V, Piperacillin, Temocillin and Ticarcillin. Penicillins are effective, e.g., against Gram-positive bacteria, facultative anaerobes, e.g., *Streptococcus*,

Borrelia, and Treponema. Penicillins are believed to inhibit bacterial cell wall synthesis by disrupting synthesis of the peptidoglycan layer of bacterial cell walls.

[295] Penicillin combinations include, but are not limited to, Amoxicillin/clavulanate, Ampicillin/sulbactam, Piperacillin/tazobactam, and Ticarcillin/clavulanate.

[296] Polypeptide antibiotics include, but are not limited to, Bacitracin, Colistin, and Polymyxin B and E. Polypeptide Antibiotics are effective, e.g., against Gram-negative bacteria. Certain polypeptide antibiotics are believed to inhibit isoprenyl pyrophosphate involved in synthesis of the peptidoglycan layer of bacterial cell walls, while others destabilize the bacterial outer membrane by displacing bacterial counter-ions.

[297] Quinolones and Fluoroquinolone include, but are not limited to, Ciprofloxacin, Enoxacin, Gatifloxacin, Gemifloxacin, Levofloxacin, Lomefloxacin, Moxifloxacin, Nalidixic acid, Norfloxacin, Ofloxacin, Trovafloxacin, Grepafloxacin, Sparfloxacin, and Temafloxacin. Quinolones/Fluoroquinolone are effective, e.g., against *Streptococcus* and *Neisseria*. Quinolones/Fluoroquinolone are believed to inhibit the bacterial DNA gyrase or topoisomerase IV, thereby inhibiting DNA replication and transcription.

[298] Sulfonamides include, but are not limited to, Mafenide, Sulfacetamide, Sulfadiazine, Silver sulfadiazine, Sulfadimethoxine, Sulfamethizole, Sulfamethoxazole, Sulfanilimide, Sulfasalazine, Sulfisoxazole, Trimethoprim-Sulfamethoxazole (Co-trimoxazole), and Sulfonamidochrysoidine. Sulfonamides are believed to inhibit folate synthesis by competitive inhibition of dihydropteroate synthetase, thereby inhibiting nucleic acid synthesis.

[299] Tetracyclines include, but are not limited to, Demeclocycline, Doxycycline, Minocycline, Oxytetracycline, and Tetracycline. Tetracyclines are effective, e.g., against Gram-negative bacteria. Tetracyclines are believed to bind to the bacterial 30S ribosomal subunit thereby inhibiting bacterial protein synthesis.

[300] Anti-mycobacterial compounds include, but are not limited to, Clofazimine, Dapsone, Capreomycin, Cycloserine, Ethambutol, Ethionamide, Isoniazid, Pyrazinamide, Rifampicin, Rifabutin, Rifapentine, and Streptomycin.

[301] Suitable antibiotics also include arsphenamine, chloramphenicol, fosfomycin, fusidic acid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, tigecycline, tinidazole, trimethoprim amoxicillin/clavulanate, ampicillin/sulbactam, amphotericin

ristocetin, azithromycin, bacitracin, buforin II, carbomycin, cecropin PI, clarithromycin, erythromycins, furazolidone, fusidic acid, Na fusidate, gramicidin, imipenem, indolicidin, josamycin, magainan II, metronidazole, nitroimidazoles, mikamycin, mutacin B-Ny266, mutacin B-JHI 140, mutacin J-T8, nisin, nisin A, novobiocin, oleandomycin, ostreogrycin, piperacillin/tazobactam, pristnamycin, ramoplanin, ranalexin, reuterin, rifaximin, rosamicin, rosaramicin, spectinomycin, spiramycin, staphylomycin, streptogramin, streptogramin A, synergistin, taurolidine, teicoplanin, telithromycin, ticarcillin/clavulanic acid, triacetyloleandomycin, tylosin, tyrocidin, tyrothricin, vancomycin, vemamycin, and virginiamycin.

Administration

[302] In certain aspects, provided herein is a method of delivering a pharmaceutical composition described herein (e.g., a pharmaceutical composition comprising *Harryflintia acetispora* mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof) to a subject. In some embodiments of the methods provided herein, the pharmaceutical composition is administered in conjunction with the administration of an additional therapeutic agent. In some embodiments, the pharmaceutical composition comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, co-formulated with the additional therapeutic agent. In some embodiments, the pharmaceutical composition comprising mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, is co-administered with the additional therapeutic agent. In some embodiments, the additional therapeutic agent is administered to the subject before administration of the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 55 minutes before, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 hours before, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days before). In some embodiments, the additional therapeutic agent is administered to the subject after administration of the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 55 minutes after, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 hours after, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days after). In some embodiments, the same mode of delivery is used to deliver both the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs),

bacteria, or any combination thereof, and the additional therapeutic agent. In some embodiments, different modes of delivery are used to administer the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, and the additional therapeutic agent. For example, in some embodiments the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, is administered orally while the additional therapeutic agent is administered via injection (e.g., an intravenous, intramuscular and/or intratumoral injection). In some embodiments, the pharmaceutical composition described herein is administered once a day. In some embodiments, the pharmaceutical composition described herein is administered twice a day. In some embodiments, the pharmaceutical composition described herein is formulated for a daily dose. In some embodiments, the pharmaceutical composition described herein is formulated for twice a day dose, wherein each dose is half of the daily dose.

[303] In certain embodiments, the pharmaceutical compositions and dosage forms described herein can be administered in conjunction with any other conventional anti-cancer treatment, such as, for example, radiation therapy and surgical resection of the tumor. These treatments may be applied as necessary and/or as indicated and may occur before, concurrent with or after administration of the pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, or dosage forms described herein.

[304] The dosage regimen can be any of a variety of methods and amounts, and can be determined by one skilled in the art according to known clinical factors. As is known in the medical arts, dosages for any one patient can depend on many factors, including the subject's species, size, body surface area, age, sex, immunocompetence, and general health, the particular microorganism to be administered, duration and route of administration, the kind and stage of the disease, for example, tumor size, and other compounds such as drugs being administered concurrently or near-concurrently. In addition to the above factors, such levels can be affected by the infectivity of the microorganism, and the nature of the microorganism, as can be determined by one skilled in the art. In the present methods, appropriate minimum dosage levels of microorganisms can be levels sufficient for the microorganism to survive, grow and replicate. The dose of a pharmaceutical composition that comprises mEVs (such as smEVs and/or pmEVs), bacteria, or any combination thereof, described herein may be appropriately set or adjusted in accordance with the dosage form,

the route of administration, the degree or stage of a target disease, and the like. For example, the general effective dose of the agents may range between 0.01 mg/kg body weight/day and 1000 mg/kg body weight/day, between 0.1 mg/kg body weight/day and 1000 mg/kg body weight/day, 0.5 mg/kg body weight/day and 500 mg/kg body weight/day, 1 mg/kg body weight/day and 100 mg/kg body weight/day, or between 5 mg/kg body weight/day and 50 mg/kg body weight/day. The effective dose may be 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 500, or 1000 mg/kg body weight/day or more, but the dose is not limited thereto.

[305] In some embodiments, the dose administered to a subject is sufficient to prevent disease (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease), delay its onset, or slow or stop its progression, or relieve one or more symptoms of the disease. One skilled in the art will recognize that dosage will depend upon a variety of factors including the strength of the particular agent (e.g., therapeutic agent) employed, as well as the age, species, condition, and body weight of the subject. The size of the dose will also be determined by the route, timing, and frequency of administration as well as the existence, nature, and extent of any adverse side-effects that might accompany the administration of a particular therapeutic agent and the desired physiological effect.

[306] Suitable doses and dosage regimens can be determined by conventional range-finding techniques known to those of ordinary skill in the art. Generally, treatment is initiated with smaller dosages, which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. An effective dosage and treatment protocol can be determined by routine and conventional means, starting e.g., with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. Animal studies are commonly used to determine the maximal tolerable dose ("MTD") of bioactive agent per kilogram weight. Those skilled in the art regularly extrapolate doses for efficacy, while avoiding toxicity, in other species, including humans.

[307] In accordance with the above, in therapeutic applications, the dosages of the therapeutic agents used in accordance with the invention vary depending on the active agent, the age, weight, and clinical condition of the recipient patient, and the experience and judgment of the clinician or practitioner administering the therapy, among other factors affecting the selected dosage. For example, for cancer treatment, the dose should be sufficient to result in slowing, and preferably regressing, the growth of a tumor and most

preferably causing complete regression of the cancer, or reduction in the size or number of metastases. As another example, the dose should be sufficient to result in slowing of progression of the disease for which the subject is being treated, and preferably amelioration of one or more symptoms of the disease for which the subject is being treated.

[308] Separate administrations can include any number of two or more administrations, including two, three, four, five or six administrations. One skilled in the art can readily determine the number of administrations to perform or the desirability of performing one or more additional administrations according to methods known in the art for monitoring therapeutic methods and other monitoring methods provided herein. Accordingly, the methods provided herein include methods of providing to the subject one or more administrations of a pharmaceutical composition, where the number of administrations can be determined by monitoring the subject, and, based on the results of the monitoring, determining whether or not to provide one or more additional administrations. Deciding on whether or not to provide one or more additional administrations can be based on a variety of monitoring results.

[309] The time period between administrations can be any of a variety of time periods. The time period between administrations can be a function of any of a variety of factors, including monitoring steps, as described in relation to the number of administrations, the time period for a subject to mount an immune response. In one example, the time period can be a function of the time period for a subject to mount an immune response; for example, the time period can be more than the time period for a subject to mount an immune response, such as more than about one week, more than about ten days, more than about two weeks, or more than about a month; in another example, the time period can be less than the time period for a subject to mount an immune response, such as less than about one week, less than about ten days, less than about two weeks, or less than about a month.

[310] In some embodiments, the delivery of an additional therapeutic agent in combination with the pharmaceutical composition described herein reduces the adverse effects and/or improves the efficacy of the additional therapeutic agent.

[311] The effective dose of an additional therapeutic agent described herein is the amount of the additional therapeutic agent that is effective to achieve the desired therapeutic response for a particular subject, composition, and mode of administration, with the least toxicity to the subject. The effective dosage level can be identified using the methods described herein and will depend upon a variety of pharmacokinetic factors including the

activity of the particular compositions or agents administered, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors well known in the medical arts. In general, an effective dose of an additional therapeutic agent will be the amount of the additional therapeutic agent which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

The toxicity of an additional therapeutic agent is the level of adverse effects experienced by the subject during and following treatment. Adverse events associated with additional therapy toxicity can include, but are not limited to, abdominal pain, acid indigestion, acid reflux, allergic reactions, alopecia, anaphylaxis, anemia, anxiety, lack of appetite, arthralgias, asthenia, ataxia, azotemia, loss of balance, bone pain, bleeding, blood clots, low blood pressure, elevated blood pressure, difficulty breathing, bronchitis, bruising, low white blood cell count, low red blood cell count, low platelet count, cardiotoxicity, cystitis, hemorrhagic cystitis, arrhythmias, heart valve disease, cardiomyopathy, coronary artery disease, cataracts, central neurotoxicity, cognitive impairment, confusion, conjunctivitis, constipation, coughing, cramping, cystitis, deep vein thrombosis, dehydration, depression, diarrhea, dizziness, dry mouth, dry skin, dyspepsia, dyspnea, edema, electrolyte imbalance, esophagitis, fatigue, loss of fertility, fever, flatulence, flushing, gastric reflux, gastroesophageal reflux disease, genital pain, granulocytopenia, gynecomastia, glaucoma, hair loss, hand-foot syndrome, headache, hearing loss, heart failure, heart palpitations, heartburn, hematoma, hemorrhagic cystitis, hepatotoxicity, hyperamylasemia, hypercalcemia, hyperchloremia, hyperglycemia, hyperkalemia, hyperlipasemia, hypermagnesemia, hypernatremia, hyperphosphatemia, hyperpigmentation, hypertriglyceridemia, hyperuricemia, hypoalbuminemia, hypocalcemia, hypochloremia, hypoglycemia, hypokalemia, hypomagnesemia, hyponatremia, hypophosphatemia, impotence, infection, injection site reactions, insomnia, iron deficiency, itching, joint pain, kidney failure, leukopenia, liver dysfunction, memory loss, menopause, mouth sores, mucositis, muscle pain, myalgias, myelosuppression, myocarditis, neutropenic fever, nausea, nephrotoxicity, neutropenia, nosebleeds, numbness, ototoxicity, pain, palmar-plantar erythrodysesthesia, pancytopenia, pericarditis, peripheral neuropathy, pharyngitis,

photophobia, photosensitivity, pneumonia, pneumonitis, proteinuria, pulmonary embolus, pulmonary fibrosis, pulmonary toxicity, rash, rapid heart beat, rectal bleeding, restlessness, rhinitis, seizures, shortness of breath, sinusitis, thrombocytopenia, tinnitus, urinary tract infection, vaginal bleeding, vaginal dryness, vertigo, water retention, weakness, weight loss, weight gain, and xerostomia. In general, toxicity is acceptable if the benefits to the subject achieved through the therapy outweigh the adverse events experienced by the subject due to the therapy.

Metabolic disorders

[312] In some embodiments, the methods and pharmaceutical compositions described herein relate to the treatment or prevention of a metabolic disease or disorder a, such as type II diabetes, impaired glucose tolerance, insulin resistance, obesity, hyperglycemia, hyperinsulinemia, fatty liver, non-alcoholic steatohepatitis, hypercholesterolemia, hypertension, hyperlipoproteinemia, hyperlipidemia, hypertriglyceridemia, ketoacidosis, hypoglycemia, thrombotic disorders, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), Nonalcoholic Steatohepatitis (NASH) or a related disease. In some embodiments, the related disease is cardiovascular disease, atherosclerosis, kidney disease, nephropathy, diabetic neuropathy, diabetic retinopathy, sexual dysfunction, dermatopathy, dyspepsia, or edema. In some embodiments, the methods and pharmaceutical compositions described herein relate to the treatment of Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH).

[313] The methods described herein can be used to treat any subject in need thereof. As used herein, a "subject in need thereof" includes any subject that has a metabolic disease or disorder, as well as any subject with an increased likelihood of acquiring a such a disease or disorder.

[314] The pharmaceutical compositions described herein can be used, for example, for preventing or treating (reducing, partially or completely, the adverse effects of) a metabolic disease, such as type II diabetes, impaired glucose tolerance, insulin resistance, obesity, hyperglycemia, hyperinsulinemia, fatty liver, non-alcoholic steatohepatitis, hypercholesterolemia, hypertension, hyperlipoproteinemia, hyperlipidemia, hypertriglyceridemia, ketoacidosis, hypoglycemia, thrombotic disorders, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), Nonalcoholic Steatohepatitis (NASH), or a related disease. In some embodiments, the related disease is cardiovascular disease, atherosclerosis,

kidney disease, nephropathy, diabetic neuropathy, diabetic retinopathy, sexual dysfunction, dermatopathy, dyspepsia, or edema.

Immune disorders

[315] In some embodiments, the methods and pharmaceutical compositions described herein relate to the treatment or prevention of a disease or disorder associated a pathological immune response, such as an autoimmune disease, an allergic reaction and/or an inflammatory disease. In some embodiments, the disease or disorder is an inflammatory bowel disease (e.g., Crohn's disease or ulcerative colitis). In some embodiments, the disease or disorder is psoriasis. In some embodiments, the disease or disorder is atopic dermatitis.

[316] The methods described herein can be used to treat any subject in need thereof. As used herein, a "subject in need thereof" includes any subject that has a disease or disorder associated with a pathological immune response (e.g., an inflammatory bowel disease), as well as any subject with an increased likelihood of acquiring a such a disease or disorder.

[317] The pharmaceutical compositions described herein can be used, for example, as a pharmaceutical composition for preventing or treating (reducing, partially or completely, the adverse effects of) an autoimmune disease, such as chronic inflammatory bowel disease, systemic lupus erythematosus, psoriasis, muckle-wells syndrome, rheumatoid arthritis, multiple sclerosis, or Hashimoto's disease; an allergic disease, such as a food allergy, pollenosis, or asthma; an infectious disease, such as an infection with *Clostridium difficile*; an inflammatory disease such as a TNF-mediated inflammatory disease (e.g., an inflammatory disease of the gastrointestinal tract, such as pouchitis, a cardiovascular inflammatory condition, such as atherosclerosis, or an inflammatory lung disease, such as chronic obstructive pulmonary disease); a pharmaceutical composition for suppressing rejection in organ transplantation or other situations in which tissue rejection might occur; a supplement, food, or beverage for improving immune functions; or a reagent for suppressing the proliferation or function of immune cells.

[318] In some embodiments, the methods provided herein are useful for the treatment of inflammation. In certain embodiments, the inflammation of any tissue and organs of the body, including musculoskeletal inflammation, vascular inflammation, neural inflammation, digestive system inflammation, ocular inflammation, inflammation of the reproductive system, and other inflammation, as discussed below.

[319] Immune disorders of the musculoskeletal system include, but are not limited, to those conditions affecting skeletal joints, including joints of the hand, wrist, elbow, shoulder, jaw, spine, neck, hip, knee, ankle, and foot, and conditions affecting tissues connecting muscles to bones such as tendons. Examples of such immune disorders, which may be treated with the methods and compositions described herein include, but are not limited to, arthritis (including, for example, osteoarthritis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, acute and chronic infectious arthritis, arthritis associated with gout and pseudogout, and juvenile idiopathic arthritis), tendonitis, synovitis, tenosynovitis, bursitis, fibrositis (fibromyalgia), epicondylitis, myositis, and osteitis (including, for example, Paget's disease, osteitis pubis, and osteitis fibrosa cystica).

[320] Ocular immune disorders refers to a immune disorder that affects any structure of the eye, including the eye lids. Examples of ocular immune disorders which may be treated with the methods and compositions described herein include, but are not limited to, blepharitis, blepharochalasis, conjunctivitis, dacryoadenitis, keratitis, keratoconjunctivitis sicca (dry eye), scleritis, trichiasis, and uveitis

[321] Examples of nervous system immune disorders which may be treated with the methods and compositions described herein include, but are not limited to, encephalitis, Guillain-Barre syndrome, meningitis, neuromyotonia, narcolepsy, multiple sclerosis, myelitis and schizophrenia. Examples of inflammation of the vasculature or lymphatic system which may be treated with the methods and compositions described herein include, but are not limited to, atherosclerosis, arthritis, phlebitis, vasculitis, and lymphangitis.

[322] Examples of digestive system immune disorders which may be treated with the methods and pharmaceutical compositions described herein include, but are not limited to, cholangitis, cholecystitis, enteritis, enterocolitis, gastritis, gastroenteritis, inflammatory bowel disease, ileitis, and proctitis. Inflammatory bowel diseases include, for example, certain art-recognized forms of a group of related conditions. Several major forms of inflammatory bowel diseases are known, with Crohn's disease (regional bowel disease, e.g., inactive and active forms) and ulcerative colitis (e.g., inactive and active forms) the most common of these disorders. In addition, the inflammatory bowel disease encompasses irritable bowel syndrome, microscopic colitis, lymphocytic-plasmacytic enteritis, coeliac disease, collagenous colitis, lymphocytic colitis and eosinophilic enterocolitis. Other less common forms of IBD include indeterminate colitis, pseudomembranous colitis (necrotizing colitis), ischemic inflammatory bowel disease, Behcet's disease, sarcoidosis, scleroderma,

IBD-associated dysplasia, dysplasia associated masses or lesions, and primary sclerosing cholangitis.

[323] Examples of reproductive system immune disorders which may be treated with the methods and pharmaceutical compositions described herein include, but are not limited to, cervicitis, chorioamnionitis, endometritis, epididymitis, omphalitis, oophoritis, orchitis, salpingitis, tubo-ovarian abscess, urethritis, vaginitis, vulvitis, and vulvodynia.

[324] The methods and pharmaceutical compositions described herein may be used to treat autoimmune conditions having an inflammatory component. Such conditions include, but are not limited to, acute disseminated alopecia universalis, Behcet's disease, Chagas' disease, chronic fatigue syndrome, dysautonomia, encephalomyelitis, ankylosing spondylitis, aplastic anemia, hidradenitis suppurativa, autoimmune hepatitis, autoimmune oophoritis, celiac disease, Crohn's disease, diabetes mellitus type 1, giant cell arteritis, goodpasture's syndrome, Grave's disease, Guillain-Barre syndrome, Hashimoto's disease, Henoch-Schönlein purpura, Kawasaki's disease, lupus erythematosus, microscopic colitis, microscopic polyarteritis, mixed connective tissue disease, Muckle-Wells syndrome, multiple sclerosis, myasthenia gravis, opsoclonus myoclonus syndrome, optic neuritis, orofacial dermatitis, pemphigus, polyarteritis nodosa, polymyalgia, rheumatoid arthritis, Reiter's syndrome, Sjögren's syndrome, temporal arteritis, Wegener's granulomatosis, warm autoimmune hemolytic anemia, interstitial cystitis, Lyme disease, morphea, psoriasis, sarcoidosis, scleroderma, ulcerative colitis, and vitiligo.

[325] The methods and pharmaceutical compositions described herein may be used to treat T-cell mediated hypersensitivity diseases having an inflammatory component. Such conditions include, but are not limited to, contact hypersensitivity, contact dermatitis (including that due to poison ivy), urticaria, skin allergies, respiratory allergies (hay fever, allergic rhinitis, house dust mite allergy) and gluten-sensitive enteropathy (Celiac disease).

[326] Other immune disorders which may be treated with the methods and pharmaceutical compositions include, for example, appendicitis, dermatitis, dermatomyositis, endocarditis, fibrositis, gingivitis, glossitis, hepatitis, hidradenitis suppurativa, iritis, laryngitis, mastitis, myocarditis, nephritis, otitis, pancreatitis, parotitis, pericarditis, peritonitis, pharyngitis, pleuritis, pneumonitis, prostatitis, pyelonephritis, and stomatitis, transplant rejection (involving organs such as kidney, liver, heart, lung, pancreas (e.g., islet cells), bone marrow, cornea, small bowel, skin allografts, skin homografts, and heart valve xenografts, sepsis, and graft vs host disease), acute pancreatitis, chronic

pancreatitis, acute respiratory distress syndrome, Sexary's syndrome, congenital adrenal hyperplasia, nonsuppurative thyroiditis, hypercalcemia associated with cancer, pemphigus, bullous dermatitis herpetiformis, severe erythema multiforme, exfoliative dermatitis, seborrheic dermatitis, seasonal or perennial allergic rhinitis, bronchial asthma, contact dermatitis, atopic dermatitis, drug hypersensitivity reactions, allergic conjunctivitis, keratitis, herpes zoster ophthalmicus, iritis and oiridocyclitis, chorioretinitis, optic neuritis, symptomatic sarcoidosis, fulminating or disseminated pulmonary tuberculosis chemotherapy, idiopathic thrombocytopenic purpura in adults, secondary thrombocytopenia in adults, acquired (autoimmune) haemolytic anemia, leukaemia and lymphomas in adults, acute leukaemia of childhood, regional enteritis, autoimmune vasculitis, multiple sclerosis, chronic obstructive pulmonary disease, solid organ transplant rejection, sepsis. Preferred treatments include treatment of transplant rejection, rheumatoid arthritis, psoriatic arthritis, multiple sclerosis, Type 1 diabetes, asthma, inflammatory bowel disease, systemic lupus erythematosus, psoriasis, chronic obstructive pulmonary disease, and inflammation accompanying infectious conditions (e.g., sepsis).

Cancer

[327] In some embodiments, the methods and pharmaceutical compositions described herein relate to the treatment of cancer. In some embodiments, any cancer can be treated using the methods described herein. Examples of cancers that may be treated by methods and pharmaceutical compositions described herein include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma;

acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and roblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse;

malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

[328] In some embodiments, the methods and pharmaceutical compositions provided herein relate to the treatment of a leukemia. The term "leukemia" includes broadly progressive, malignant diseases of the hematopoietic organs/systems and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Non-limiting examples of leukemia diseases include, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemiac leukemia, basophilic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, undifferentiated cell leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, and promyelocytic leukemia.

[329] In some embodiments, the methods and pharmaceutical compositions provided herein relate to the treatment of a carcinoma. The term "carcinoma" refers to a malignant growth made up of epithelial cells tending to infiltrate the surrounding tissues, and/or resist physiological and non-physiological cell death signals and gives rise to metastases. Non-limiting exemplary types of carcinomas include, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatousum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform

carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiennoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberos carcinoma, verrucous carcinoma, carcinoma villosum, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypernephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, nasopharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, and carcinoma scroti.

[330] In some embodiments, the methods and pharmaceutical compositions provided herein relate to the treatment of a sarcoma. The term "sarcoma" generally refers to a tumor which is made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar, heterogeneous, or homogeneous substance. Sarcomas include, but are not limited to, chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, endometrial sarcoma, stromal sarcoma, Ewing's sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumor sarcoma, granulocytic sarcoma, Hodgkin's sarcoma,

idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectatic sarcoma.

[331] Additional exemplary neoplasias that can be treated using the methods and pharmaceutical compositions described herein include Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insuloma, malignant carcinoid, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, plasmacytoma, colorectal cancer, rectal cancer, and adrenal cortical cancer.

[332] In some embodiments, the cancer treated is a melanoma. The term "melanoma" is taken to mean a tumor arising from the melanocytic system of the skin and other organs. Non-limiting examples of melanomas are Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, nodular melanoma subungual melanoma, and superficial spreading melanoma.

[333] In some embodiments, the cancer comprises breast cancer (e.g., triple negative breast cancer).

[334] In some embodiments, the cancer comprises colorectal cancer (e.g., microsatellite stable (MSS) colorectal cancer).

[335] In some embodiments, the cancer comprises renal cell carcinoma.

[336] In some embodiments, the cancer comprises lung cancer (e.g., non small cell lung cancer).

[337] In some embodiments, the cancer comprises bladder cancer.

[338] In some embodiments, the cancer comprises gastroesophageal cancer.

[339] In some embodiments, the cancer comprises a solid tumor.

[340] Particular categories of tumors that can be treated using methods and pharmaceutical compositions described herein include lymphoproliferative disorders, breast cancer, ovarian cancer, prostate cancer, cervical cancer, endometrial cancer, bone cancer,

liver cancer, stomach cancer, colon cancer, pancreatic cancer, cancer of the thyroid, head and neck cancer, cancer of the central nervous system, cancer of the peripheral nervous system, skin cancer, kidney cancer, as well as metastases of all the above. Particular types of tumors include hepatocellular carcinoma, hepatoma, hepatoblastoma, rhabdomyosarcoma, esophageal carcinoma, thyroid carcinoma, ganglioblastoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, Ewing's tumor, leiomyosarcoma, rhabdotherliosarcoma, invasive ductal carcinoma, papillary adenocarcinoma, melanoma, pulmonary squamous cell carcinoma, basal cell carcinoma, adenocarcinoma (well differentiated, moderately differentiated, poorly differentiated or undifferentiated), bronchioloalveolar carcinoma, renal cell carcinoma, hypernephroma, hypernephroid adenocarcinoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, lung carcinoma including small cell, non-small and large cell lung carcinoma, bladder carcinoma, glioma, astrocyoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, retinoblastoma, neuroblastoma, colon carcinoma, rectal carcinoma, hematopoietic malignancies including all types of leukemia and lymphoma including: acute myelogenous leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, mast cell leukemia, multiple myeloma, myeloid lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, plasmacytoma, colorectal cancer, and rectal cancer.

[341] Cancers treated in certain embodiments also include precancerous lesions, e.g., actinic keratosis (solar keratosis), moles (dysplastic nevi), actinic cheilitis (farmer's lip), cutaneous horns, Barrett's esophagus, atrophic gastritis, dyskeratosis congenita, sideropenic dysphagia, lichen planus, oral submucous fibrosis, actinic (solar) elastosis and cervical dysplasia.

[342] Cancers treated in some embodiments include non-cancerous or benign tumors, e.g., of endodermal, ectodermal or mesenchymal origin, including, but not limited to, cholangioma, colonic polyp, adenoma, papilloma, cystadenoma, liver cell adenoma, hydatidiform mole, renal tubular adenoma, squamous cell papilloma, gastric polyp, hemangioma, osteoma, chondroma, lipoma, fibroma, lymphangioma, leiomyoma, rhabdomyoma, astrocytoma, nevus, meningioma, and ganglioneuroma.

Dysbiosis

[343] In recent years, it has become increasingly clear that the gut microbiome (also called the “gut microbiota”) can have a significant impact on an individual’s health through microbial activity and influence (local and/or distal) on immune and other cells of the host (Walker, W.A., Dysbiosis. The Microbiota in Gastrointestinal Pathophysiology. Chapter 25. 2017; Weiss and Thierry, Mechanisms and consequences of intestinal dysbiosis. *Cellular and Molecular Life Sciences*. (2017) 74(16):2959-2977. Zurich Open Repository and Archive).

[344] A healthy host-gut microbiome homeostasis is sometimes referred to as a “eubiosis” or “normobiosis,” whereas a detrimental change in the host microbiome composition and/or its diversity can lead to an unhealthy imbalance in the microbiome, or a “dysbiosis” (Hooks and O’Malley. Dysbiosis and its discontents. *American Society for Microbiology*. Oct 2017. Vol. 8. Issue 5. mBio 8:e01492-17). Dysbiosis, and associated local or distal host inflammatory or immune effects, may occur where microbiome homeostasis is lost or diminished, resulting in: increased susceptibility to pathogens; altered host bacterial metabolic activity; induction of host proinflammatory activity and/or reduction of host anti-inflammatory activity. Such effects are mediated in part by interactions between host immune cells (e.g., T cells, dendritic cells, mast cells, NK cells, intestinal epithelial lymphocytes (IEC), macrophages and phagocytes) and cytokines, and other substances released by such cells and other host cells.

[345] A dysbiosis may occur within the gastrointestinal tract (a “gastrointestinal dysbiosis” or “gut dysbiosis”) or may occur outside the lumen of the gastrointestinal tract (a “distal dysbiosis”). Gastrointestinal dysbiosis is often associated with a reduction in integrity of the intestinal epithelial barrier, reduced tight junction integrity and increased intestinal permeability. Citi, S. Intestinal Barriers protect against disease, *Science* 359:1098-99 (2018); Srinivasan et al., TEER measurement techniques for in vitro barrier model systems. *J. Lab. Autom.* 20:107-126 (2015). A gastrointestinal dysbiosis can have physiological and immune effects within and outside the gastrointestinal tract.

[346] The presence of a dysbiosis has been associated with a wide variety of diseases and conditions including: infection, cancer, autoimmune disorders (e.g., systemic lupus erythematosus (SLE)) or inflammatory disorders (e.g., functional gastrointestinal disorders such as inflammatory bowel disease (IBD), ulcerative colitis, and Crohn’s disease), neuroinflammatory diseases (e.g., multiple sclerosis), transplant disorders (e.g.,

graft-versus-host disease), fatty liver disease, type I diabetes, rheumatoid arthritis, Sjögren's syndrome, celiac disease, cystic fibrosis, chronic obstructive pulmonary disorder (COPD), and other diseases and conditions associated with immune dysfunction. Lynch et al., The Human Microbiome in Health and Disease, *N. Engl. J. Med.* 375:2369-79 (2016), Carding et al., Dysbiosis of the gut microbiota in disease, *Microb. Ecol. Health Dis.* (2015); 26: 10: 3402/mehd.v26.2619; Levy et al, Dysbiosis and the Immune System, *Nature Reviews Immunology* 17:219 (April 2017).

[347] Exemplary pharmaceutical compositions disclosed herein can treat a dysbiosis and its effects by modifying the immune activity present at the site of dysbiosis. As described herein, such compositions can modify a dysbiosis via effects on host immune cells, resulting in, e.g., an increase in secretion of anti-inflammatory cytokines and/or a decrease in secretion of pro-inflammatory cytokines, reducing inflammation in the subject recipient or via changes in metabolite production.

[348] Exemplary pharmaceutical compositions disclosed herein that are useful for treatment of disorders associated with a dysbiosis contain one or more types of immunomodulatory bacteria (e.g., anti-inflammatory bacteria), mEVs (microbial extracellular vesicles; such as smEVs and/or pmEVs) derived from such bacteria, or any combination thereof. Such compositions are capable of affecting the recipient host's immune function, in the gastrointestinal tract, and/or a systemic effect at distal sites outside the subject's gastrointestinal tract.

[349] Exemplary pharmaceutical compositions disclosed herein that are useful for treatment of disorders associated with a dysbiosis contain a population of *Harryflintia acetispora* bacteria of a single bacterial species (e.g., a single strain) (e.g., anti-inflammatory bacteria) mEVs (such as smEVs and/or pmEVs) derived from such bacteria, or any combination thereof. Such compositions are capable of affecting the recipient host's immune function, in the gastrointestinal tract, and /or a systemic effect at distal sites outside the subject's gastrointestinal tract.

[350] In some embodiments, pharmaceutical compositions containing an isolated population of *Harryflintia acetispora* bacteria (e.g., anti-inflammatory bacterial cells), mEVs (such as smEVs and/or pmEVs) derived from such bacteria, or any combination thereof, are administered (e.g., orally) to a mammalian recipient in an amount effective to treat a dysbiosis and one or more of its effects in the recipient. The dysbiosis may be a gastrointestinal tract dysbiosis or a distal dysbiosis.

[351] In another embodiment, pharmaceutical compositions of the instant invention can treat a gastrointestinal dysbiosis and one or more of its effects on host immune cells, resulting in an increase in secretion of anti-inflammatory cytokines and/or a decrease in secretion of pro-inflammatory cytokines, reducing inflammation in the subject recipient.

[352] In another embodiment, the pharmaceutical compositions can treat a gastrointestinal dysbiosis and one or more of its effects by modulating the recipient immune response via cellular and cytokine modulation to reduce gut permeability by increasing the integrity of the intestinal epithelial barrier.

[353] In another embodiment, the pharmaceutical compositions can treat a distal dysbiosis and one or more of its effects by modulating the recipient immune response at the site of dysbiosis via modulation of host immune cells.

[354] Other exemplary pharmaceutical compositions are useful for treatment of disorders associated with a dysbiosis, which compositions contain one or more types of *Harryflintia acetispora* bacteria, mEVs (such as smEVs and/or pmEVs) derived from such bacteria, or any combination thereof, capable of altering the relative proportions of host immune cell subpopulations, e.g., subpopulations of T cells, immune lymphoid cells, dendritic cells, NK cells and other immune cells, or the function thereof, in the recipient.

[355] Other exemplary pharmaceutical compositions are useful for treatment of disorders associated with a dysbiosis, which compositions contain a population of *Harryflintia acetispora* bacteria, mEVs, or any combination thereof, of a single bacterial species capable of altering the relative proportions of immune cell subpopulations, e.g., T cell subpopulations, immune lymphoid cells, NK cells and other immune cells, or the function thereof, in the recipient subject.

[356] In some embodiments, the invention provides methods of treating a gastrointestinal dysbiosis and one or more of its effects by orally administering to a subject in need thereof a pharmaceutical composition described herein which alters the microbiome population existing at the site of the dysbiosis. The pharmaceutical composition can contain one or more types of *Harryflintia acetispora* bacteria, mEVs, or any combination thereof, or a population of *Harryflintia acetispora* bacteria, mEVs, or any combination thereof, of a single bacterial species (e.g., a single strain).

[357] In some embodiments, the invention provides methods of treating a distal dysbiosis and one or more of its effects by orally administering to a subject in need thereof a pharmaceutical composition described herein which alters the subject's immune response

outside the gastrointestinal tract. The pharmaceutical composition can contain one or more types of *Harryflintia acetispora* bacteria, mEVs, or any combination thereof; or a population of *Harryflintia acetispora* bacteria, mEVs, or any combination thereof, of a single bacterial species (e.g., a single strain).

[358] In exemplary embodiments, pharmaceutical compositions useful for treatment of disorders associated with a dysbiosis stimulate secretion of one or more anti-inflammatory cytokines by host immune cells. Anti-inflammatory cytokines include, but are not limited to, IL-10, IL-13, IL-9, IL-4, IL-5, TGF β , and combinations thereof. In other exemplary embodiments, pharmaceutical compositions useful for treatment of disorders associated with a dysbiosis that decrease (e.g., inhibit) secretion of one or more pro-inflammatory cytokines by host immune cells. Pro-inflammatory cytokines include, but are not limited to, IFN γ , IL-12p70, IL-1 α , IL-6, IL-8, MCP1, MIP1 α , MIP1 β , TNF α , and combinations thereof. Other exemplary cytokines are known in the art and are described herein.

[359] In another aspect, the invention provides a method of treating or preventing a disorder associated with a dysbiosis in a subject in need thereof, comprising administering (e.g., orally administering) to the subject a therapeutic composition in the form of a probiotic or medical food comprising bacteria, mEVs, or any combination thereof, in an amount sufficient to alter the microbiome at a site of the dysbiosis, such that the disorder associated with the dysbiosis is treated.

[360] In another embodiment, a therapeutic composition of the instant invention in the form of a probiotic or medical food may be used to prevent or delay the onset of a dysbiosis in a subject at risk for developing a dysbiosis.

Other Diseases and Disorders

[361] In some embodiments, the methods and pharmaceutical compositions described herein relate to the treatment of liver diseases. Such diseases include, but are not limited to, Alagille Syndrome, Alcohol-Related Liver Disease, Alpha-1 Antitrypsin Deficiency, Autoimmune Hepatitis, Benign Liver Tumors, Biliary Atresia, Cirrhosis, Galactosemia, Gilbert Syndrome, Hemochromatosis, Hepatitis A, Hepatitis B, Hepatitis C, Hepatic Encephalopathy, Intrahepatic Cholestasis of Pregnancy (ICP), Lysosomal Acid Lipase Deficiency (LAL-D), Liver Cysts, Liver Cancer, Newborn Jaundice, Primary Biliary

Cholangitis (PBC), Primary Sclerosing Cholangitis (PSC), Reye Syndrome, Type I Glycogen Storage Disease, and Wilson Disease.

[362] The methods and pharmaceutical compositions described herein may be used to treat neurodegenerative and neurological diseases. In certain embodiments, the neurodegenerative and/or neurological disease is Parkinson's disease, Alzheimer's disease, prion disease, Huntington's disease, motor neuron diseases (MND), spinocerebellar ataxia, spinal muscular atrophy, dystonia, idiopathic intracranial hypertension, epilepsy, nervous system disease, central nervous system disease, movement disorders, multiple sclerosis, encephalopathy, peripheral neuropathy or post-operative cognitive dysfunction.

Methods of Making Enhanced Bacteria

[363] In certain aspects, provided herein are methods of making engineered bacteria for the production of the mEVs (such as smEVs and/or pmEVs), bacteria for pharmaceutical compositions, or any combination thereof, described herein. In some embodiments, the engineered bacteria are modified to enhance certain desirable properties. For example, in some embodiments, the engineered bacteria are modified to enhance the immunomodulatory and/or therapeutic effect of the mEVs (such as smEVs and/or pmEVs), bacteria for pharmaceutical compositions, or any combination thereof, (e.g., either alone or in combination with another therapeutic agent), to reduce toxicity and/or to improve bacterial and/or mEV (such as smEV and/or pmEV) manufacturing (e.g., higher oxygen tolerance, improved freeze-thaw tolerance, shorter generation times). The engineered bacteria may be produced using any technique known in the art, including but not limited to site-directed mutagenesis, transposon mutagenesis, knock-outs, knock-ins, polymerase chain reaction mutagenesis, chemical mutagenesis, ultraviolet light mutagenesis, transformation (chemically or by electroporation), phage transduction, directed evolution, CRISPR/Cas9, or any combination thereof.

[364] In some embodiments of the methods provided herein, the bacterium is modified by directed evolution. In some embodiments, the directed evolution comprises exposure of the bacterium to an environmental condition and selection of bacterium with improved survival and/or growth under the environmental condition. In some embodiments, the method comprises a screen of mutagenized bacteria using an assay that identifies enhanced bacterium. In some embodiments, the method further comprises mutagenizing the bacteria (e.g., by exposure to chemical mutagens and/or UV radiation) or exposing them to a

therapeutic agent (e.g., antibiotic) followed by an assay to detect bacteria having the desired phenotype (e.g., an *in vivo* assay, an *ex vivo* assay, or an *in vitro* assay).

EXAMPLES

Example 1: Cultivation and storage conditions for *Harryflintia acetispora*

Anaerobic Tryptic Soy Broth (TSB) medium:

Composition (g/L):

- | | |
|--|--------|
| 1. Pancreatic Digest of Casein | 17.0 g |
| 2. Peptic Digest of Soybean Meal | 3.0 g |
| 3. Dipotassium Phosphate K ₂ HPO ₄ | 2.5 g |
| 4. Yeast Extract | 5.0 g |
| 5. Dextrose | 2.5 g |
| 6. Sodium Chloride | 5.0 g |
| 7. L-Cysteine-HCL | 0.5 g |
| 8. FeCl ₂ | 0.05 g |

Final pH 6.8 +/- 0.3 at 25 degrees C.

Reducing agent 100× preparation:

- Dissolve 5 g of L-Cysteine-HCl in 100 ml of H₂O
- Add 0.5 g of FeCl₂
- Store solution under anaerobic conditions

Anaerobic Brucella blood agar plates, Anaerobe systems #AS-141.

Anaerobic glycerol (GSS) as cryoprotectant, Anaerobe Systems #AS-9045:

Composition (g/L):

- Glycerol 400 g
- Magnesium Sulfate, Heptahydrate 0.1 g
- Potassium Phosphate, Monobasic 0.2 g
- Potassium Chloride 0.2 g
- Sodium Phosphate, Dibasic 1.15 g

6. Sodium Chloride 3.0 g
7. Sodium Thioglycollate 1.0 g
8. L-Cysteine (25.0% solution) 2.0 mL

Mix anaerobic glycerol with bacterial suspension 1:1 by volume, snap-freeze and store at -80 °C; Incubation at 37°C for 1-3 days; Anaerobic conditions: a) for agar plates in Mitsubishi anaerobic jars with anaerobic gas pack; b) for liquid culture in closed anaerobic Hungate or Balch test tubes.

Example 2: Oral Delivery of *Harryflintia acetispora* smEVs Strain A in CT26 Tumor Studies

First Representative Oncology Study

[365] Female 8 week old BALB/c mice were acquired from Taconic Biosciences and allowed to acclimate at a vivarium for 3 weeks. On Day 0, mice were anesthetized with isoflurane, and inoculated subcutaneously on the left flank with 1.0×10^5 CT-26 cells (0.1mL) prepared in PBS and Corning (GFR) Phenol Red-Free Matrigel (1:1). Mice were allowed to rest for 9 days post CT-26 inoculation to allow formation of palpable tumors. On Day 9, tumors were measured using a sliding digital caliper to collect length and width in measurements (in millimeters) to calculate estimated tumor volume ($(L \times W \times W)/2 = TV_{mm^3}$). Mice were randomized into different treatment groups with a total of 9 or 10 mice per group. Randomization was done to balance all treatment groups, allowing each group to begin treatment with a similar average tumor volume and standard deviation. Dosing began on Day 10, and ended on Day 22 for 13 consecutive days of dosing. Mice were orally dosed BID with *Harryflintia acetispora* Strain A smEVs, or Q4D intraperitoneally with 200ug anti-mouse PD-1 antibody. Body weight and tumor measurements were collected on a MWF (Monday-Wednesday-Friday) schedule. Dose of smEVs was determined by particle count by NTA.

[366] The Day 22 Tumor Volume Summary in Fig. 1 compares *Harryflintia acetispora* (2e11) smEV (growth media containing glucose) and *Harryflintia acetispora* (2e11) smEV (growth media 2 containing NAG) against a negative control (Vehicle PBS), and positive control (anti-PD-1). Both *Harryflintia acetispora* treatment groups compared to Vehicle PBS showed statistically significant efficacy and are not significantly different than anti-PD-1. The Tumor Volume Curves in Fig. 2 show similar growth trends for both *Harryflintia acetispora* groups and anti-PD-1, along with sustained efficacy after 13 days of treatment.

Second representative Oncology Study

[367] Female 8 week old BALB/c mice were acquired from Taconic Biosciences and allowed to acclimate at a vivarium for 1 week. On Day 0, mice were anesthetized with isoflurane, and inoculated subcutaneously on the left flank with 1.0×10^5 CT-26 cells (0.1mL) prepared in PBS and Corning (GFR) Phenol Red-Free Matrigel (1:1). Mice were allowed to rest for 9 days post CT-26 inoculation to allow formation of palpable tumors. On Day 9, tumors were measured using a sliding digital caliper to collect length and width in measurements (in millimeters) to calculate estimated tumor volume $((L \times W \times W)/2) = TV_{mm^3}$). Mice were randomized into different treatment groups with a total of 9 mice per group. Randomization was done to balance all treatment groups, allowing each group to begin treatment with a similar average tumor volume and standard deviation. Dosing began on Day 10, and ended on Day 23 for 14 consecutive days of dosing. Mice were orally dosed BID with *Harryflintia acetispora* Strain A smEVs, or Q4D intraperitoneally with 200ug anti-mouse PD-1 antibody. Body weight and tumor measurements were collected on a MWF schedule. Dose of smEVs was determined by particle count by NTA.

[368] The Day 23 Tumor Volume Summary in Fig. 3 compares *Harryflintia acetispora* smEV (2e11) against a negative control (Vehicle PBS), and positive control (anti-PD-1). The *Harryflintia acetispora* smEV (2e11) treatment groups compared to Vehicle PBS shows statistically significant efficacy compared to Vehicle (PBS), and is not significantly different than anti-PD-1. Although *Harryflintia acetispora* is not significantly different than anti-PD-1, the data distribution is much tighter, and average tumor volume is smaller for *Harryflintia acetispora* smEV. The Tumor Growth Curve in Fig. 4 shows sustained efficacy of *Harryflintia acetispora* smEV treatment groups over 14 days of treatment similar to anti-PD-1.

Third Representative Oncology Study

[369] Female 8 week old BALB/c mice were acquired from Taconic Biosciences and allowed to acclimate at a vivarium for 1 week. On Day 0, mice were anesthetized with isoflurane, and inoculated subcutaneously on the left flank with 1.0×10^5 CT-26 cells (0.1mL) prepared in PBS and Corning (GFR) Phenol Red-Free Matrigel (1:1). Mice were allowed to rest for 9 days post CT-26 inoculation to allow formation of palpable tumors. On Day 9, tumors were measured using a sliding digital caliper to collect length and width in measurements (in millimeters) to calculate estimated tumor volume $((L \times W \times W)/2) = TV_{mm^3}$). Mice were randomized into different treatment groups with a total of 9 mice per group. Randomization was

done to balance all treatment groups, allowing each group to begin treatment with a similar average tumor volume and standard deviation. Dosing began on Day 10, and ended on Day 21 for 12 consecutive days of dosing. Mice were orally dosed QD and BID respectively with *Harryflintia acetispora* Strain A smEVs, or Q4D intraperitoneally with 200ug anti-mouse PD-1 antibody. Body weight and tumor measurements were collected on a MWF schedule. Dose of smEVs was determined by particle count by NTA.

[370] The Day 21 Tumor Volume Summary in Fig. 5 on the left, compares *Harryflintia acetispora* smEVs at 3 doses (4e11, 4e9, & 4e7), administered QD for 12 days against respective negative control (Vehicle PBS) and positive control (anti-PD-1). The Tumor Volume Summary in Fig. 5 also compares *Harryflintia acetispora* smEVs at 2 doses (2e11 & 2e7) administered BID for a total daily dose of 4e11 & 4e7 respectively against their corresponding negative control (Vehicle PBS BID) and positive control (anti-PD-1). The QD *Harryflintia acetispora* smEVs treatment arms compared to Vehicle PBS show statistically significant efficacy across all (3) doses, without an evident dose effect. The BID *Harryflintia acetispora* smEVs treatment arms compared to Vehicle PBS BID show significant efficacy, also no evidence of a dose effect. This result indicates both doses BID 2e11 & 2e7 are comparable dosed BID and QD. The Tumor Growth Curve in Fig. 6 shows a sustained efficacy after 12 days of dosing similar to anti-PD-1.

Example 3: Oral Delivery of *Harryflintia acetispora* Strain A Whole Bacterium in CT26 Tumor Studies

[371] Female 8 week old BALB/c mice were acquired from Taconic Biosciences and allowed to acclimate at a vivarium for 1 week. On Day 0, mice were anesthetized with isoflurane, and inoculated subcutaneously on the left flank with 1.0e5 CT-26 cells (0.1mL) prepared in PBS and Corning (GFR) Phenol Red-Free Matrigel (1:1). Mice were allowed to rest for 9 days post CT-26 inoculation to allow formation of palpable tumors. On Day 9, tumors were measured using a sliding digital caliper to collect length and width in measurements (in millimeters) to calculate estimated tumor volume $((L \times W \times W)/2 = TV_{mm^3})$. Mice were randomized into different treatment groups with a total of (10) mice per group. Randomization was done to balance all treatment groups, allowing each group to begin treatment with a similar average tumor volume and standard deviation. Dosing began on Day 10, and ended on Day 24 for 14 consecutive days of dosing. Mice were orally dosed QD and BID respectively with *Harryflintia acetispora* Strain A microbes,

or Q4D intraperitoneally with 200ug anti-mouse PD-1 antibody. Body weight and tumor measurements were collected on a Monday-Wednesday-Friday (MWF) schedule.

[372] The Day 24 Tumor Volume Summary in Fig. 7 and the Tumor Growth Curve in Fig. 8 compare the *Harryflintia acetispora* Strain A microbes dosed at (2e11 cells), administered BID for 14 days, against respective negative control (Vehicle PBS) and positive control (anti-PD-1). The *Harryflintia acetispora* treatment arm compared to the Vehicle PBS BID showed similar tumor growth at Day 24. The positive control anti-PD1 treatment arm compared to the Vehicle PBS BID also showed similar tumor growth.

Example 4: Oral Delievery of *Harryflintia acetispora* Strain A smEVs in a Mouse Model of Delayed-Type Hypersensitivity (DTH)

[373] Delayed-type hypersensitivity (DTH) is an animal model of atopic dermatitis (or allergic contact dermatitis), as reviewed by Petersen et al. (In vivo pharmacological disease models for psoriasis and atopic dermatitis in drug discovery. Basic & Clinical Pharm & Toxicology. 2006. 99(2): 104-115; see also Irving C. Allen (ed.) Mouse Models of Innate Immunity: Methods and Protocols, Methods in Molecular Biology, 2013. vol. 1031, DOI 10.1007/978-1-62703-481-4_13). Several variations of the DTH model have been used and are well known in the art (Irving C. Allen (ed.). Mouse Models of Innate Immunity: Methods and Protocols, Methods in Molecular Biology. Vol. 1031, DOI 10.1007/978-1-62703-481-4_13, Springer Science + Business Media, LLC 2013).

KLH DTH Study:

[374] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. Mice were orally gavaged daily with *Harryflintia acetispora* Strain A smEVs or dosed intraperitoneally with dexamethasone at 1 mg/kg from days 1-8. After dosing on day 8, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 µl) in the left ear and ear thickness measurements were taken at 24 hours. Dose was determined by particle count by NTA.

[375] The 24 hour ear measurement results are shown in Fig. 9. smEVs made from *Harryflintia acetispora* were tested at two doses, 2E+11 and 2E+07, based on particles per dose. The *Harryflintia acetispora* Strain A smEVs were efficacious, showing decreased

levels of ear inflammation 24 hours after challenge. *Harryflintia acetispora* Strain A was as efficacious at the lower dose as the higher dose.

Example 5: Oral Delivery of *Harryflintia acetispora* Strain A smEVs in a Mouse Model of Delayed-Type Hypersensitivity (DTH)

[376] Delayed-type hypersensitivity (DTH) is an animal model of atopic dermatitis (or allergic contact dermatitis), as reviewed by Petersen et al. (In vivo pharmacological disease models for psoriasis and atopic dermatitis in drug discovery. Basic & Clinical Pharm & Toxicology. 2006. 99(2): 104-115; see also Irving C. Allen (ed.) Mouse Models of Innate Immunity: Methods and Protocols, Methods in Molecular Biology, 2013. vol. 1031, DOI 10.1007/978-1-62703-481-4_13). Several variations of the DTH model have been used and are well known in the art (Irving C. Allen (ed.) Mouse Models of Innate Immunity: Methods and Protocols, Methods in Molecular Biology. Vol. 1031, DOI 10.1007/978-1-62703-481-4_13, Springer Science + Business Media, LLC 2013).

KLH DTH Study:

[377] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. Mice were orally gavaged daily with *Harryflintia acetispora* Strain A smEVs or dosed intraperitoneally with dexamethasone at 1 mg/kg from days 5-8. After dosing on day 8, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 µl) in the left ear and ear thickness measurements were taken at 24 hours.

[378] The 24 hour ear measurement results are shown in Fig. 10. smEVs made from *Harryflintia acetispora* Strain A were compared at three doses, 2E+11, 2E+09 and 2E+07 (based on particles per dose). The *Harryflintia acetispora* Strain A smEVs were efficacious at all three doses, showing decreased ear inflammation 24 hours after challenge.

Example 6: *Harryflintia acetispora* Strain A smEV U937 Testing Protocol

Cell Line Preparation

1. The U937 Monocyte cell line (ATCC) was propagated in RPMI medium with added FBS HEPES, sodium pyruvate, and antibiotic. at 37°C with 5% CO₂.

2. Cells were enumerated using a cellometer with live/dead staining to determine viability.
3. Cells were diluted to a concentration of 5×10^5 cells per ml in RPMI medium with 20nM phorbol-12-myristate-13-acetate (PMA) to differentiate the monocytes into macrophage-like cells.
4. 200 microliters of cell suspension was added to each well of a 96-well plate and incubated 37°C with 5% CO₂ for 72hrs.
5. The adherent, differentiated cells were washed and incubated in fresh medium without PMA for 24hrs before experimentation.

Experimental Setup

1. smEVs were diluted to the appropriate concentration in RPMI medium without antibiotics (typically 1×10^5 - 1×10^{10}).
2. Treatment-free and TLR 2 and 4 agonist control samples are also prepared
3. The 96-well plate containing the differentiated U937 cells was washed with fresh RPMI medium without antibiotics, to remove residual antibiotics.
4. The suspension of smEVs was added to the washed plate.
5. The plate was incubated for 24hrs at 37°C with 5% CO₂.

Experimental Endpoints

1. After 24hrs of coincubation the supernatants were removed from the U937 cells into a separate 96-well plate. The cells were observed for any obvious lysis (plaques) in the wells.
2. Two treatment-free wells did not have the supernatants removed and Lysis buffer was added to the wells and incubated at 37°C for 30 minutes to lyse cells (maximum lysis control).
3. 50 microliters of each supernatant or maximum lysis control was added to a new 96-well plate and cell lysis was determined (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) per manufacturer's instructions.
4. Cytokines were measured from the supernatants using U-plex MSD plates (Meso Scale Discovery) per manufacturer's instructions.

[379] smEVs from *Harryflintia acetispora* Strain A induce cytokine production from PMA-differentiated U937 cells (Fig. 11). U937 cells were treated with *Harryflintia*

acetispora Strain A smEV at 1×10^6 - 1×10^9 concentrations as well as TLR2 (FSL) and TLR4 (LPS) agonist controls for 24hrs and cytokine production was measured. Note the stepwise increase in cytokine production. "Blank" indicates the medium control.

Example 7: *Harveyflintia acetispora* Strain A Gamma-Irradiated Whole Bacterium U937

Testing Protocol

Cell Line Preparation

1. The U937 Monocyte cell line (ATCC) was propagated in RPMI medium with added FBS HEPES, sodium pyruvate, and antibiotic. at 37°C with 5% CO₂.
2. Cells were enumerated using a cellometer with live/dead staining to determine viability.
3. Cells were diluted to a concentration of 5×10^5 cells per ml in RPMI medium with 20nM phorbol-12-myristate-13-acetate (PMA) to differentiate the monocytes into macrophage-like cells.
4. 200 microliters of cell suspension was added to each well of a 96-well plate and incubated 37°C with 5% CO₂ for 72hrs.
5. The adherent, differentiated cells were washed and incubated in fresh medium without PMA for 24hrs before experimentation.

Experimental Setup

1. Bacteria were diluted to the appropriate concentration in RPMI medium without antibiotics (typically 1×10^5 - 1×10^{10}).
2. Treatment-free and TLR 2 and 4 agonist control samples are also prepared
3. The 96-well plate containing the differentiated U937 cells was washed with fresh RPMI medium without antibiotics, to remove residual antibiotics.
4. The suspension of Bacteria was added to the washed plate.
5. The plate was incubated for 24hrs at 37°C with 5% CO₂.

Experimental Endpoints

1. After 24hrs of coinubation the supernatants were removed from the U937 cells into a separate 96-well plate. The cells were observed for any obvious lysis (plaques) in the wells.

2. Two treatment-free wells did not have the supernatants removed and Lysis buffer was added to the wells and incubated at 37°C for 30 minutes to lyse cells (maximum lysis control).
3. 50 microliters of each supernatant or maximum lysis control was added to a new 96-well plate and cell lysis was determined (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) per manufacturer's instructions.
4. Cytokines were measured from the supernatants using U-plex MSD plates (Meso Scale Discovery) per manufacturer's instructions.

[380] *Harryflintia acetispora* Strain A whole gamma-irradiated microbes induce cytokine production from PMA-differentiated U937 cells (Fig. 12). U937 cells were treated with *Harryflintia acetispora* Strain A whole gamma-irradiated microbes at 1×10^5 and 1×10^6 concentrations as well as TLR2 (FSL) and TLR4 (LPS) agonist controls for 24hrs and cytokine production was measured. "Blank" indicates the medium control.

Example 8: *Harryflintia acetispora* Strain A smEV Isolation and Enumeration

Equipment required:

Sorvall RC-5C centrifuge with SLA-3000 rotor

Optima XE-90 Ultracentrifuge by Beckman-Coulter

45Ti rotor

Sorvall wX+ Ultra Series Centrifuge by Thermo Scientific

Fiberlite F37L-8x100 rotor

1. Microbial Supernatant Collection and Filtration:

Microbes must be pelleted and filtered away from supernatant in order to recover smEVs and not microbes.

a. Pellet Microbial culture

- i. Use Sorvall RC-5C centrifuge with the SLA-3000 rotor and centrifuge culture for a minimum of 15min at a minimum of 7,000rpm.
- ii. Decant supernatant into new and sterile container.

b. Supernatant Filtration

- i. Filter supernatant through 0.2um filter.
- ii. For supernatants with poor filterability (less than 300ml of supernatant pass through filter) attach a 0.45um capsule filter ahead of the 0.2um vacuum filter.

- iii. Store 'filtered' supernatant at 4°C.
- iv. Filtered supernatant can then be concentrated using TFF.

2. Isolation of smEVs using Ultracentrifugation

Centrifuging concentrated supernatant in the ultracentrifuge will pellet smEVs isolating the smEVs from smaller biomolecules.

- i. Set speed for 200,000g, time for 1 hour, and temperature at 4°C.
- ii. When rotor has stopped, remove tubes from ultracentrifuge and gently pour off the supernatant.
- iii. Add more supernatant, balance, and centrifuge tubes again.
- iv. After all concentrated supernatant has been centrifuged, the pellets generated are referred to as 'crude' smEV pellets.
- v. Add sterile 1xPBS to pellets and place in container. Place on shaker, speed 70, in 4°C fridge overnight or longer.
- vi. Resuspend the smEV pellets with additional sterile 1xPBS.
 - 1. Store resuspended crude smEV samples at 4°C or at -80°C.

3. smEV Purification using Density Gradients

Density gradients are used for smEV purification. During ultracentrifugation, particles in the sample will move, and separate, within the graded density medium based on their 'buoyant' densities. In this way smEVs are separated from other particles, such as sugars, lipids, or other proteins, in the sample.

a. Preparation of Density Medium

- i. For smEV purification, four different percentages of the density medium (60% Optiprep) are used, a 45% layer, a 35% layer, a 25%, and a 15% layer. This will create the graded layers. A 0% layer is added at the top consisting of sterile 1xPBS.
- ii. The 45% gradient layer should contain the crude smEV sample. 5ml of sample is added to 15ml of Optiprep. If crude smEV sample is less than 5ml, bring up to volume using sterile 1xPBS.

b. Density Gradient Assembly

- i. Using a serological pipette, gently pipette the 45% gradient mixture up and down to mix. Then pipette the sample into a labeled clean and sterile ultracentrifuge tube.

- ii. Next, using a 10ml serological pipette, slowly add 13ml of 35% gradient mixture.
 - iii. Continue with 13ml of the 25% gradient mixture followed by 13ml of the 15% mixture and finally 6ml of sterile 1xPBS.
 - iv. Balance ultracentrifuge tubes with sterile 1xPBS.
 - v. Carefully place gradients in rotor and set the ultracentrifuge for 200,000g and the temperature for 4°C. Centrifuge a minimum of 16 hours.
- c. **Removing Purified smEVs from Density Gradients**
- i. Using a clean pipette, removed fraction(s) of interest and add to 15ml conical tube.
 - ii. Keep 'purified' smEV samples at 4°C.
- d. **Removing Optiprep Material from Purified smEVs**
- i. In order to clean and remove residual optiprep from smEVs, 10x volume of PBS should be added to purified smEVs.
 - ii. Set the ultracentrifuge for 200,000g and the temperature for 4°C. Centrifuge for 1 hour.
 - iii. Carefully remove tubes from ultracentrifuge and decant supernatant.
 - iv. Continue 'washing' purified mEVs until all sample has been pelleted.
 - v. Add sterile 1xPBS to purified pellets and place in container. Place on shaker, speed 70, in 4°C fridge overnight or longer.
 - vi. Resuspend the 'purified' smEV pellets with additional sterile 1xPBS.
 - 1. Store resuspended purified smEV samples at 4°C or at -80°C .

Example 9: Administering pmEV compositions to treat mouse tumor models

[381] As described in Example 2, a mouse model of cancer is generated by subcutaneously injecting a tumor cell line or patient-derived tumor sample and allowing it to engraft into healthy mice. The methods provided herein may be performed using one of several different tumor cell lines including, but not limited to: B16-F10 or B16-F10-SIY cells as an orthotopic model of melanoma, Panc02 cells as an orthotopic model of pancreatic cancer (Maletzki et al., 2008, Gut 57:483-491), LLC1 cells as an orthotopic model of lung cancer, and RM-1 as an orthotopic model of prostate cancer. As an example, but without

limitation, methods for studying the efficacy of pmEVs in the B16-F10 model are provided in depth herein.

[382] A syngeneic mouse model of spontaneous melanoma with a very high metastatic frequency is used to test the ability of bacteria to reduce tumor growth and the spread of metastases. The pmEVs chosen for this assay are compositions that may display enhanced activation of immune cell subsets and stimulate enhanced killing of tumor cells *in vitro*. The mouse melanoma cell line B16-F10 is obtained from ATCC. The cells are cultured in vitro as a monolayer in RPMI medium, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ in air. The exponentially growing tumor cells are harvested by trypsinization, washed three times with cold 1x PBS, and a suspension of 5E6 cells/ml is prepared for administration. Female C57BL/6 mice are used for this experiment. The mice are 6-8 weeks old and weigh approximately 16-20 g. For tumor development, each mouse is injected SC into the flank with 100 µl of the B16-F10 cell suspension. The mice are anesthetized by ketamine and xylazine prior to the cell transplantation. The animals used in the experiment may be started on an antibiotic treatment via instillation of a cocktail of kanamycin (0.4 mg/ml), gentamicin, (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml) and vancomycin (0.045 mg/ml) in the drinking water from day 2 to 5 and an intraperitoneal injection of clindamycin (10 mg/kg) on day 7 after tumor injection.

[383] The size of the primary flank tumor is measured with a caliper every 2-3 days and the tumor volume is calculated using the following formula: tumor volume = the tumor width × tumor length × 0.5. After the primary tumor reaches approximately 100 mm³, the animals are sorted into several groups based on their body weight. The mice are then randomly taken from each group and assigned to a treatment group. pmEV compositions are prepared as previously described. The mice are orally inoculated by gavage with approximately 7.0e+09 to 3.0e+12 pmEV particles. Alternatively, pmEVs are administered intravenously. Mice receive pmEVs daily, weekly, bi-weekly, monthly, bi-monthly, or on any other dosing schedule throughout the treatment period. Mice may be IV injected with pmEVs in the tail vein, or directly injected into the tumor. Mice can be injected with pmEVs, with or without live bacteria, and/or pmEVs with or without inactivated/weakened or killed bacteria. Mice can be injected or orally gavaged weekly or once a month. Mice may receive combinations of purified pmEVs and live bacteria to maximize tumor-killing potential. All mice are housed under specific pathogen-free conditions following approved

protocols. Tumor size, mouse weight, and body temperature are monitored every 3-4 days and the mice are humanely sacrificed 6 weeks after the B16-F10 mouse melanoma cell injection or when the volume of the primary tumor reaches 1000 mm³. Blood draws are taken weekly and a full necropsy under sterile conditions is performed at the termination of the protocol.

[384] Cancer cells can be easily visualized in the mouse B16-F10 melanoma model due to their melanin production. Following standard protocols, tissue samples from lymph nodes and organs from the neck and chest region are collected and the presence of micro- and macro-metastases is analyzed using the following classification rule. An organ is classified as positive for metastasis if at least two micro-metastatic and one macro-metastatic lesion per lymph node or organ are found. Micro-metastases are detected by staining the paraffin-embedded lymphoid tissue sections with hematoxylin-eosin following standard protocols known to one skilled in the art. The total number of metastases is correlated to the volume of the primary tumor and it is found that the tumor volume correlates significantly with tumor growth time and the number of macro- and micro-metastases in lymph nodes and visceral organs and also with the sum of all observed metastases. Twenty-five different metastatic sites are identified as previously described (Bobek V., et al., Syngeneic lymph-node-targeting model of green fluorescent protein-expressing Lewis lung carcinoma, Clin. Exp. Metastasis, 2004;21(8):705-8).

[385] The tumor tissue samples are further analyzed for tumor infiltrating lymphocytes. The CD8⁺ cytotoxic T cells can be isolated by FACS and can then be further analyzed using customized p/MHC class I microarrays to reveal their antigen specificity (see e.g., Deviren G., et al., Detection of antigen-specific T cells on p/MHC microarrays, J. Mol. Recognit., 2007 Jan-Feb;20(1):32-8). CD4⁺ T cells can be analyzed using customized p/MHC class II microarrays.

[386] At various timepoints, mice are sacrificed and tumors, lymph nodes, or other tissues may be removed for *ex vivo* flow cytometric analysis using methods known in the art. For example, tumors are dissociated using a Miltenyi tumor dissociation enzyme cocktail according to the manufacturer's instructions. Tumor weights are recorded and tumors are chopped then placed in 15ml tubes containing the enzyme cocktail and placed on ice. Samples are then placed on a gentle shaker at 37°C for 45 minutes and quenched with up to 15ml complete RPMI. Each cell suspension is strained through a 70µm filter into a 50ml falcon tube and centrifuged at 1000 rpm for 10 minutes. Cells are resuspended in FACS

buffer and washed to remove remaining debris. If necessary, samples are strained again through a second 70µm filter into a new tube. Cells are stained for analysis by flow cytometry using techniques known in the art. Staining antibodies can include anti-CD11c (dendritic cells), anti-CD80, anti-CD86, anti-CD40, anti-MHCII, anti-CD8a, anti-CD4, and anti-CD103. Other markers that may be analyzed include pan-immune cell marker CD45, T cell markers (CD3, CD4, CD8, CD25, Foxp3, T-bet, Gata3, Rorγt, Granzyme B, CD69, PD-1, CTLA-4), and macrophage/myeloid markers (CD11b, MHCII, CD206, CD40, CSF1R, PD-L1, Gr-1). In addition to immunophenotyping, serum cytokines can be analyzed including, but not limited to, TNFα, IL-17, IL-13, IL-12p70, IL12p40, IL-10, IL-6, IL-5, IL-4, IL-2, IL-1b, IFNγ, GM-CSF, G-CSF, M-CSF, MIG, IP10, MIP1b, RANTES, and MCP-1. Cytokine analysis may be carried out immune cells obtained from lymph nodes or other tissue, and/or on purified CD45+ tumor-infiltrated immune cells obtained ex vivo. Finally, immunohistochemistry is carried out on tumor sections to measure T cells, macrophages, dendritic cells, and checkpoint molecule protein expression.

[387] The same experiment is also performed with a mouse model of multiple pulmonary melanoma metastases. The mouse melanoma cell line B16-BL6 is obtained from ATCC and the cells are cultured in vitro as described above. Female C57BL/6 mice are used for this experiment. The mice are 6-8 weeks old and weigh approximately 16-20 g. For tumor development, each mouse is injected into the tail vein with 100 µl of a 2E6 cells/ml suspension of B16-BL6 cells. The tumor cells that engraft upon IV injection end up in the lungs.

[388] The mice are humanely killed after 9 days. The lungs are weighed and analyzed for the presence of pulmonary nodules on the lung surface. The extracted lungs are bleached with Fekete's solution, which does not bleach the tumor nodules because of the melanin in the B16 cells though a small fraction of the nodules is amelanotic (i.e. white). The number of tumor nodules is carefully counted to determine the tumor burden in the mice. Typically, 200-250 pulmonary nodules are found on the lungs of the control group mice (i.e. PBS gavage).

[389] The percentage tumor burden is calculated for the three treatment groups. Percentage tumor burden is defined as the mean number of pulmonary nodules on the lung surfaces of mice that belong to a treatment group divided by the mean number of pulmonary nodules on the lung surfaces of the control group mice.

[390] The tumor biopsies and blood samples are submitted for metabolic analysis via LCMS techniques or other methods known in the art. Differential levels of amino acids, sugars, lactate, among other metabolites, between test groups demonstrate the ability of the microbial composition to disrupt the tumor metabolic state.

RNA Seq to Determine Mechanism of Action

[391] Dendritic cells are purified from tumors, Peyers patches, and mesenteric lymph nodes. RNAseq analysis is carried out and analyzed according to standard techniques known to one skilled in the art (Z. Hou. *Scientific Reports*. 5(9570):doi:10.1038/srep09570 (2015)). In the analysis, specific attention is placed on innate inflammatory pathway genes including TLRs, CLRs, NLRs, and STING, cytokines, chemokines, antigen processing and presentation pathways, cross presentation, and T cell co-stimulation.

[392] Rather than being sacrificed, some mice may be rechallenged with tumor cell injection into the contralateral flank (or other area) to determine the impact of the immune system's memory response on tumor growth.

Example 10: Administering pmEVs to treat mouse tumor models in combination with PD-1 or PD-L1 inhibition

[393] To determine the efficacy of pmEVs in tumor mouse models, in combination with PD-1 or PD-L1 inhibition, a mouse tumor model may be used as described above.

[394] pmEVs are tested for their efficacy in the mouse tumor model, either alone or in combination with whole bacterial cells and with or without anti-PD-1 or anti-PD-L1. pmEVs, bacterial cells, and/or anti-PD-1 or anti-PD-L1 are administered at varied time points and at varied doses. For example, on day 10 after tumor injection, or after the tumor volume reaches 100mm³, the mice are treated with pmEVs alone or in combination with anti-PD-1 or anti-PD-L1.

[395] Mice may be administered pmEVs orally, intravenously, or intratumorally. For example, some mice are intravenously injected with anywhere between 7.0e+09 to 3.0e+12 pmEV particles. While some mice receive pmEVs through i.v. injection, other mice may receive pmEVs through intraperitoneal (i.p.) injection, subcutaneous (s.c.) injection, nasal route administration, oral gavage, or other means of administration. Some mice may receive pmEVs every day (e.g., starting on day 1), while others may receive pmEVs at alternative intervals (e.g., every other day, or once every three days). Groups of mice may be administered a pharmaceutical composition of the invention comprising a mixture of pmEVs

and bacterial cells. For example, the composition may comprise pmEV particles and whole bacteria in a ratio from 1:1 (pmEVs: bacterial cells) to 1×10^{12} :1 (pmEVs: bacterial cells).

[396] Alternatively, some groups of mice may receive between 1×10^4 and 5×10^9 bacterial cells in an administration separate from, or comingled with, the pmEV administration. As with the pmEVs, bacterial cell administration may be varied by route of administration, dose, and schedule. The bacterial cells may be live, dead, or weakened. The bacterial cells may be harvested fresh (or frozen) and administered, or they may be irradiated or heat-killed prior to administration with the pmEVs. Some groups of mice are also injected with effective doses of checkpoint inhibitor. For example, mice receive 100 μ g anti-PD-L1 mAB (clone 10f.9g2, BioXCell) or another anti-PD-1 or anti-PD-L1 mAB in 100 μ l PBS, and some mice receive vehicle and/or other appropriate control (e.g., control antibody). Mice are injected with mABs 3, 6, and 9 days after the initial injection. To assess whether checkpoint inhibition and pmEV immunotherapy have an additive anti-tumor effect, control mice receiving anti-PD-1 or anti-PD-L1 mABs are included to the standard control panel. Primary (tumor size) and secondary (tumor infiltrating lymphocytes and cytokine analysis) endpoints are assessed, and some groups of mice may be rechallenged with a subsequent tumor cell inoculation to assess the effect of treatment on memory response.

Example 11: Labeling bacterial pmEVs

[397] pmEVs may be labeled in order to track their biodistribution *in vivo* and to quantify and track cellular localization in various preparations and in assays conducted with mammalian cells. For example, pmEVs may be radio-labeled, incubated with dyes, fluorescently labeled, luminescently labeled, or labeled with conjugates containing metals and isotopes of metals.

[398] For example, pmEVs may be incubated with dyes conjugated to functional groups such as NHS-ester, click-chemistry groups, streptavidin or biotin. The labeling reaction may occur at a variety of temperatures for minutes or hours, and with or without agitation or rotation. The reaction may then be stopped by adding a reagent such as bovine serum albumin (BSA), or similar agent, depending on the protocol, and free or unbound dye molecule removed by ultra-centrifugation, filtration, centrifugal filtration, column affinity purification or dialysis. Additional washing steps involving wash buffers and vortexing or agitation may be employed to ensure complete removal of free dyes molecules such as described in Su Chul Jang et al, Small. 11, No.4, 456-461(2017).

[399] Optionally, pmEVs may be concentrated to 5.0 E12 particle/ml (300ug) and diluted up to 1.8mo using 2X concentrated PBS buffer pH 8.2 and pelleted by centrifugation at 165,000 x g at 4 C using a benchtop ultracentrifuge. The pellet is resuspended in 300ul 2X PBS pH 8.2 and an NHS-ester fluorescent dye is added at a final concentration of 0.2mM from a 10mM dye stock (dissolved in DMSO). The sample is gently agitated at 24°C for 1.5 hours, and then incubated overnight at 4°C. Free non-reacted dye is removed by 2 repeated steps of dilution/pelleting as described above, using 1X PBS buffer, and resuspending in 300ul final volume.

[400] Fluorescently labeled pmEVs are detected in cells or organs, or in *in vitro* and/or *ex vivo* samples by confocal microscopy, nanoparticle tracking analysis, flow cytometry, fluorescence activated cell sorting (FACs) or fluorescent imaging system such as the Odyssey CLx LICOR (see e.g., Wiklander et al. 2015. J. Extracellular Vesicles. 4:10.3402/jev.v4.26316). Additionally, fluorescently labeled pmEVs are detected in whole animals and/or dissected organs and tissues using an instrument such as the IVIS spectrum CT (Perkin Elmer) or Pearl Imager, as in H-I. Choi, et al. *Experimental & Molecular Medicine*. 49: e330 (2017).

[401] pmEVs may be labeled with conjugates containing metals and isotopes of metals using the protocols described above. Metal-conjugated pmEVs may be administered *in vivo* to animals. Cells may then be harvested from organs at various time-points, and analyzed *ex vivo*. Alternatively, cells derived from animals, humans, or immortalized cell lines may be treated with metal-labelled pmEVs *in vitro* and cells subsequently labelled with metal-conjugated antibodies and phenotyped using a Cytometry by Time of Flight (CyTOF) instrument such as the Helios CyTOF (Fluidigm) or imaged and analyzed using an Imaging Mass Cytometry instrument such as the Hyperion Imaging System (Fluidigm). Additionally, pmEVs may be labelled with a radioisotope to track the pmEVs biodistribution (see, e.g., Miller et al., *Nanoscale*. 2014 May 7;6(9):4928-35).

Example 12: Transmission electron microscopy to visualize bacterial pmEVs

[402] pmEVs are prepared from bacteria batch cultures. Transmission electron microscopy (TEM) may be used to visualize purified bacterial pmEVs (S. Bin Park, et al. PLoS ONE. 6(3):e17629 (2011). pmEVs are mounted onto 300- or 400-mesh-size carbon-coated copper grids (Electron Microscopy Sciences, USA) for 2 minutes and washed with deionized water. pmEVs are negatively stained using 2% (w/v) uranyl acetate for 20 sec – 1

min. Copper grids are washed with sterile water and dried. Images are acquired using a transmission electron microscope with 100-120 kV acceleration voltage. Stained pmEVs appear between 20-600 nm in diameter and are electron dense. 10-50 fields on each grid are screened.

Example 13: Profiling pmEV composition and content

[403] pmEVs may be characterized by any one of various methods including, but not limited to, NanoSight characterization, SDS-PAGE gel electrophoresis, Western blot, ELISA, liquid chromatography-mass spectrometry and mass spectrometry, dynamic light scattering, lipid levels, total protein, lipid to protein ratios, nucleic acid analysis and/or zeta potential.

NanoSight Characterization of pmEVs

[404] Nanoparticle tracking analysis (NTA) is used to characterize the size distribution of purified bacterial pmEVs. Purified pmEV preparations are run on a NanoSight machine (Malvern Instruments) to assess pmEV size and concentration.

SDS-PAGE Gel Electrophoresis

[405] To identify the protein components of purified pmEVs, samples are run on a gel, for example a Bolt Bis-Tris Plus 4-12% gel (Thermo-Fisher Scientific), using standard techniques. Samples are boiled in 1x SDS sample buffer for 10 minutes, cooled to 4°C, and then centrifuged at 16,000 x g for 1 min. Samples are then run on a SDS-PAGE gel and stained using one of several standard techniques (e.g., Silver staining, Coomassie Blue, Gel Code Blue) for visualization of bands.

Western blot analysis

[406] To identify and quantify specific protein components of purified pmEVs, pmEV proteins are separated by SDS-PAGE as described above and subjected to Western blot analysis (Cvjetkovic et al., *Sci. Rep.* 6, 36338 (2016)) and are quantified via ELISA.

pmEV proteomics and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Mass Spectrometry (MS)

[407] Proteins present in pmEVs are identified and quantified by Mass Spectrometry techniques. pmEV proteins may be prepared for LC-MS/MS using standard techniques including protein reduction using dithiotreitol solution (DTT) and protein digestion using enzymes such as LysC and trypsin as described in Erickson et al, 2017 (Molecular Cell, VOLUME 65, ISSUE 2, P361-370, JANUARY 19, 2017). Alternatively,

peptides are prepared as described by Liu et al. 2010 (JOURNAL OF BACTERIOLOGY, June 2010, p. 2852–2860 Vol. 192, No. 11), Kieselbach and Oscarsson 2017 (Data Brief. 2017 Feb; 10: 426–431.), Vildhede et al, 2018 (Drug Metabolism and Disposition February 8, 2018). Following digestion, peptide preparations are run directly on liquid chromatography and mass spectrometry devices for protein identification within a single sample. For relative quantitation of proteins between samples, peptide digests from different samples are labeled with isobaric tags using the iTRAQ Reagent-8plex Multiplex Kit (Applied Biosystems, Foster City, CA) or TMT 10plex and 11plex Label Reagents (Thermo Fischer Scientific, San Jose, CA, USA). Each peptide digest is labeled with a different isobaric tag and then the labeled digests are combined into one sample mixture. The combined peptide mixture is analyzed by LC-MS/MS for both identification and quantification. A database search is performed using the LC-MS/MS data to identify the labeled peptides and the corresponding proteins. In the case of isobaric labeling, the fragmentation of the attached tag generates a low molecular mass reporter ion that is used to obtain a relative quantitation of the peptides and proteins present in each pmEV.

[408] Additionally, metabolic content is ascertained using liquid chromatography techniques combined with mass spectrometry. A variety of techniques exist to determine metabolomic content of various samples and are known to one skilled in the art involving solvent extraction, chromatographic separation and a variety of ionization techniques coupled to mass determination (Roberts et al 2012 Targeted Metabolomics. Curr Protoc Mol Biol. 30: 1-24; Dettmer et al 2007, Mass spectrometry-based metabolomics. Mass Spectrom Rev. 26(1):51-78). As a non-limiting example, a LC-MS system includes a 4000 QTRAP triple quadrupole mass spectrometer (AB SCIEX) combined with 1100 Series pump (Agilent) and an HTS PAL autosampler (Leap Technologies). Media samples or other complex metabolic mixtures (~10 µL) are extracted using nine volumes of 74.9:24.9:0.2 (v/v/v) acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8, Isotec; and phenylalanine-d8, Cambridge Isotope Laboratories). Standards may be adjusted or modified depending on the metabolites of interest. The samples are centrifuged (10 minutes, 9,000g, 4 °C), and the supernatants (10 µL) are submitted to LCMS by injecting the solution onto the HILIC column (150 × 2.1 mm, 3 µm particle size). The column is eluted by flowing a 5% mobile phase [10mM ammonium formate, 0.1% formic acid in water] for 1 minute at a rate of 250uL/minute followed by a linear gradient over 10

minutes to a solution of 40% mobile phase [acetonitrile with 0.1% formic acid]. The ion spray voltage is set to 4.5 kV and the source temperature is 450 °C.

[409] The data are analyzed using commercially available software like Multiquant 1.2 from AB SCIEX for mass spectrum peak integration. Peaks of interest should be manually curated and compared to standards to confirm the identity of the peak. Quantitation with appropriate standards is performed to determine the number of metabolites present in the initial media, after bacterial conditioning and after tumor cell growth. A non-targeted metabolomics approach may also be used using metabolite databases, such as but not limited to the NIST database, for peak identification.

Dynamic light scattering (DLS)

[410] DLS measurements, including the distribution of particles of different sizes in different pmEV preparations are taken using instruments such as the DynaPro NanoStar (Wyatt Technology) and the Zetasizer Nano ZS (Malvern Instruments).

Lipid Levels

[411] Lipid levels are quantified using FM4-64 (Life Technologies), by methods similar to those described by A.J. McBroom *et al. J Bacteriol* 188:5385-5392. and A. Frias, *et al. Microb Ecol.* 59:476-486 (2010). Samples are incubated with FM4-64 (3.3 µg/mL in PBS for 10 minutes at 37°C in the dark). After excitation at 515 nm, emission at 635 nm is measured using a Spectramax M5 plate reader (Molecular Devices). Absolute concentrations are determined by comparison of unknown samples to standards (such as palmitoyllecylphosphatidylglycerol (POPG) vesicles) of known concentrations. Lipidomics can be used to identify the lipids present in the pmEVs.

Total Protein

[412] Protein levels are quantified by standard assays such as the Bradford and BCA assays. The Bradford assays are run using Quick Start Bradford 1x Dye Reagent (Bio-Rad), according to manufacturer's protocols. BCA assays are run using the Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific). Absolute concentrations are determined by comparison to a standard curve generated from BSA of known concentrations. Alternatively, protein concentration can be calculated using the Beer-Lambert equation using the sample absorbance at 280nm (A280) as measured on a Nanodrop spectrophotometer (Thermo-Fisher Scientific). In addition, proteomics may be used to identify proteins in the sample.

Lipid:Protein Ratios

[413] Lipid:protein ratios are generated by dividing lipid concentrations by protein concentrations. These provide a measure of the purity of vesicles as compared to free protein in each preparation.

Nucleic acid analysis

[414] Nucleic acids are extracted from pmEVs and quantified using a Qubit fluorometer. Size distribution is assessed using a BioAnalyzer and the material is sequenced.

Zeta Potential

[415] The zeta potential of different preparations are measured using instruments such as the Zetasizer ZS (Malvern Instruments).

Example 14: In vitro screening of pmEVs for enhanced activation of CD8+ T cell killing when incubated with tumor cells

[416] *In vitro* methods for screening pmEVs that can activate CD8+ T cell killing of tumor cells are described. Briefly, DCs are isolated from human PBMCs or mouse spleens, using techniques known in the art, and incubated *in vitro* with single-strain pmEVs, mixtures of pmEVs, and/or appropriate controls. In addition, CD8+ T cells are obtained from human PBMCs or mouse spleens using techniques known in the art, for example the magnetic bead-based Mouse CD8a+ T Cell Isolation Kit and the magnetic bead-based Human CD8+ T Cell Isolation Kit (both from Miltenyi Biotec, Cambridge, MA). After incubation of DCs with pmEVs for some time (e.g., for 24-hours), or incubation of DCs with pmEV-stimulated epithelial cells, pmEVs are removed from the cell culture with PBS washes and 100ul of fresh media with antibiotics is added to each well, and 200,000 T cells are added to each experimental well in the 96-well plate. Anti-CD3 antibody is added at a final concentration of 2ug/ml. Co-cultures are then allowed to incubate at 37°C for 96 hours under normal oxygen conditions.

[417] For example, approximately 72 hours into the coculture incubation, tumor cells are plated for use in the assay using techniques known in the art. For example, 50,000 tumor cells/well are plated per well in new 96-well plates. Mouse tumor cell lines used may include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. After completion of the 96-hour co-culture, 100 µl of the CD8+ T cell and DC mixture is transferred to wells containing tumor cells. Plates are incubated for 24 hours at 37°C under

normal oxygen conditions. Staurosporine may be used as negative control to account for cell death.

[418] Following this incubation, flow cytometry is used to measure tumor cell death and characterize immune cell phenotype. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well. Cytotoxic CD8+ T cell phenotype may be characterized by the following methods: a) concentration of supernatant granzyme B, IFN γ and TNF α in the culture supernatant as described below, b) CD8+ T cell surface expression of activation markers such as DC69, CD25, CD154, PD-1, gamma/delta TCR, Foxp3, T-bet, granzyme B, c) intracellular cytokine staining of IFN γ , granzyme B, TNF α in CD8+ T cells. CD4+ T cell phenotype may also be assessed by intracellular cytokine staining in addition to supernatant cytokine concentration including IFN γ , TNF α , IL-12, IL-4, IL-5, IL-17, IL-10, chemokines etc.

[419] As an additional measure of CD8+ T cell activation, 100 μ l of culture supernatant is removed from wells following the 96-hour incubation of T cells with DCs and is analyzed for secreted cytokines, chemokines, and growth factors using the multiplexed Luminex Magpix. Kit (EMD Millipore, Darmstadt, Germany). Briefly, the wells are pre-wet with buffer, and 25 μ l of 1x antibody-coated magnetic beads are added and 2x 200 μ l of wash buffer are performed in every well using the magnet. 50 μ l of Incubation buffer, 50 μ l of diluent and 50 μ l of samples are added and mixed via shaking for 2hrs at room temperature in the dark. The beads are then washed twice with 200 μ l wash buffer. 100 μ l of 1X biotinylated detector antibody is added and the suspension is incubated for 1 hour with shaking in the dark. Two, 200 μ l washes are then performed with wash buffer. 100 μ l of 1x SAV-RPE reagent is added to each well and is incubated for 30 min at RT in the dark. Three 200 μ l washes are performed and 125 μ l of wash buffer is added with 2-3 min shaking occurs. The wells are then submitted for analysis in the Luminex xMAP system.

[420] Standards allow for careful quantitation of the cytokines including GM-CSF, IFN- γ , IFN- α , IFN- β IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12 (p40/p70), IL-17, IL-23, IP-10, KC, MCP-1, MIG, MIP1 α , TNF α , and VEGF. These cytokines are assessed in samples of both mouse and human origin. Increases in these cytokines in the bacterial treated samples indicate enhanced production of proteins and cytokines from the host. Other variations on this assay examining specific cell types ability to release cytokines are assessed by acquiring these cells through sorting methods and are

recognized by one of ordinary skill in the art. Furthermore, cytokine mRNA is also assessed to address cytokine release in response to an pmEV composition. These changes in the cells of the host stimulate an immune response similarly to in vivo response in a cancer microenvironment.

[421] This CD8+ T cell stimulation protocol may be repeated using combinations of purified pmEVs and live bacterial strains to maximize immune stimulation potential.

Example 15: In vitro screening of pmEVs for enhanced tumor cell killing by PBMCs

[422] Various methods may be used to screen pmEVs for the ability to stimulate PBMCs, which in turn activate CD8+ T cells to kill tumor cells. For example, PBMCs are isolated from heparinized venous blood from healthy human donors by ficoll-paque gradient centrifugation for mouse or human blood, or with Lympholyte Cell Separation Media (Cedarlane Labs, Ontario, Canada) from mouse blood. PBMCs are incubated with single-strain pmEVs, mixtures of pmEVs, and appropriate controls. In addition, CD8+ T cells are obtained from human PBMCs or mouse spleens. After the 24-hour incubation of PBMCs with pmEVs, pmEVs are removed from the cells using PBS washes. 100ul of fresh media with antibiotics is added to each well. An appropriate number of T cells (e.g., 200,000 T cells) are added to each experimental well in the 96-well plate. Anti-CD3 antibody is added at a final concentration of 2ug/ml. Co-cultures are then allowed to incubate at 37° C for 96 hours under normal oxygen conditions.

[423] For example, 72 hours into the coculture incubation, 50,000 tumor cells/well are plated per well in new 96-well plates. Mouse tumor cell lines used include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. After completion of the 96-hour co-culture, 100 µl of the CD8+ T cell and PBMC mixture is transferred to wells containing tumor cells. Plates are incubated for 24 hours at 37° C under normal oxygen conditions. Staurosporine is used as negative control to account for cell death.

[424] Following this incubation, flow cytometry is used to measure tumor cell death and characterize immune cell phenotype. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well. Cytotoxic CD8+ T cell phenotype may be characterized by the following methods:
a) concentration of supernatant granzyme B, IFN γ and TNF α in the culture supernatant as

described below, b) CD8+ T cell surface expression of activation markers such as DC69, CD25, CD154, PD-1, gamma/delta TCR, Foxp3, T-bet, granzyme B, c) intracellular cytokine staining of IFN γ , granzyme B, TNF α in CD8+ T cells. CD4+ T cell phenotype may also be assessed by intracellular cytokine staining in addition to supernatant cytokine concentration including IFN γ , TNF α , IL-12, IL-4, IL-5, IL-17, IL-10, chemokines etc.

[425] As an additional measure of CD8+ T cell activation, 100 μ l of culture supernatant is removed from wells following the 96-hour incubation of T cells with DCs and is analyzed for secreted cytokines, chemokines, and growth factors using the multiplexed Luminex Magpix. Kit (EMD Millipore, Darmstadt, Germany). Briefly, the wells are pre-wet with buffer, and 25 μ l of 1x antibody-coated magnetic beads are added and 2x 200 μ l of wash buffer are performed in every well using the magnet. 50 μ l of Incubation buffer, 50 μ l of diluent and 50 μ l of samples are added and mixed via shaking for 2hrs at room temperature in the dark. The beads are then washed twice with 200 μ l wash buffer. 100 μ l of 1X biotinylated detector antibody is added and the suspension is incubated for 1 hour with shaking in the dark. Two, 200 μ l washes are then performed with wash buffer. 100 μ l of 1x SAV-RPE reagent is added to each well and is incubated for 30 min at RT in the dark. Three 200 μ l washes are performed and 125 μ l of wash buffer is added with 2-3 min shaking occurs. The wells are then submitted for analysis in the Luminex xMAP system.

[426] Standards allow for careful quantitation of the cytokines including GM-CSF, IFN- γ , IFN- α , IFN- β IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12 (p40/p70), IL-17, IL-23, IP-10, KC, MCP-1, MIG, MIP1 α , TNF α , and VEGF. These cytokines are assessed in samples of both mouse and human origin. Increases in these cytokines in the bacterial treated samples indicate enhanced production of proteins and cytokines from the host. Other variations on this assay examining specific cell types ability to release cytokines are assessed by acquiring these cells through sorting methods and are recognized by one of ordinary skill in the art. Furthermore, cytokine mRNA is also assessed to address cytokine release in response to an pmEV composition. These changes in the cells of the host stimulate an immune response similarly to in vivo response in a cancer microenvironment.

[427] This PBMC stimulation protocol may be repeated using combinations of purified pmEVs with or without combinations of live, dead, or inactivated/weakened bacterial strains to maximize immune stimulation potential.

Example 16: In vitro detection of pmEVs in antigen-presenting cells

[428] Dendritic cells in the lamina propria constantly sample live bacteria, dead bacteria, and microbial products in the gut lumen by extending their dendrites across the gut epithelium, which is one way that pmEVs produced by bacteria in the intestinal lumen may directly stimulate dendritic cells. The following methods represent a way to assess the differential uptake of pmEVs by antigen-presenting cells. Optionally, these methods may be applied to assess immunomodulatory behavior of pmEVs administered to a patient.

[429] Dendritic cells (DCs) are isolated from human or mouse bone marrow, blood, or spleens according to standard methods or kit protocols (e.g., Inaba K, Swiggard WJ, Steinman RM, Romani N, Schuler G, 2001. Isolation of dendritic cells. Current Protocols in Immunology. Chapter 3:Unit3.7).

[430] To evaluate pmEV entrance into and/or presence in DCs, 250,000 DCs are seeded on a round cover slip in complete RPMI-1640 medium and are then incubated with pmEVs from single bacterial strains or combinations pmEVs at various ratios. Purified pmEVs may be labeled with fluorochromes or fluorescent proteins. After incubation for various timepoints (e.g., 1 hour, 2 hours), the cells are washed twice with ice-cold PBS and detached from the plate using trypsin. Cells are either allowed to remain intact or are lysed. Samples are then processed for flow cytometry. Total internalized pmEVs are quantified from lysed samples, and percentage of cells that uptake pmEVs is measured by counting fluorescent cells. The methods described above may also be performed in substantially the same manner using macrophages or epithelial cell lines (obtained from the ATCC) in place of DCs.

Example 17: In vitro screening of pmEVs with an enhanced ability to activate NK cell killing when incubated with target cells

[431] To demonstrate the ability of the selected pmEV compositions to elicit potent NK cell cytotoxicity to tumor cells, the following *in vitro* assay is used. Briefly, mononuclear cells from heparinized blood are obtained from healthy human donors. Optionally, an expansion step to increase the numbers of NK cells is performed as previously described (e.g., see Somanschi et al., *J Vis Exp.* 2011;(48):2540). The cells may be adjusted to a concentration of ,cells/ml in RPMI-1640 medium containing 5% human serum. The PMNC cells are then labeled with appropriate antibodies and NK cells are isolated through FACS as CD3-/CD56+ cells and are ready for the subsequent cytotoxicity

assay. Alternatively, NK cells are isolated using the autoMACs instrument and NK cell isolation kit following manufacturer's instructions (Miltenyl Biotec).

[432] NK cells are counted and plated in a 96 well format with 20,000 or more cells per well, and incubated with single-strain pmEVs, with or without addition of antigen presenting cells (e.g., monocytes derived from the same donor), pmEVs from mixtures of bacterial strains, and appropriate controls. After 5-24 hours incubation of NK cells with pmEVs, pmEVs are removed from cells with PBS washes, NK cells are resuspended in 10 mL fresh media with antibiotics and are added to 96-well plates containing 20,000 target tumor cells/well. Mouse tumor cell lines used include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. Plates are incubated for 2-24 hours at 37°C under normal oxygen conditions. Staurosporine is used as negative control to account for cell death.

[433] Following this incubation, flow cytometry is used to measure tumor cell death using methods known in the art. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well.

[434] This NK cell stimulation protocol may be repeated using combinations of purified pmEVs and live bacterial strains to maximize immune stimulation potential.

Example 18: Using *in vitro* immune activation assays to predict *in vivo* cancer immunotherapy efficacy of pmEV compositions

[435] *In vitro* immune activation assays identify pmEVs that are able to stimulate dendritic cells, which in turn activate CD8+ T cell killing. Therefore, the *in vitro* assays described above are used as a predictive screen of a large number of candidate pmEVs for potential immunotherapy activity. pmEVs that display enhanced stimulation of dendritic cells, enhanced stimulation of CD8+ T cell killing, enhanced stimulation of PBMC killing, and/or enhanced stimulation of NK cell killing, are preferentially chosen for *in vivo* cancer immunotherapy efficacy studies.

Example 19: Determining the biodistribution of pmEVs when delivered orally to mice

[436] Wild-type mice (e.g., C57BL/6 or BALB/c) are orally inoculated with the pmEV composition of interest to determine the *in vivo* biodistribution profile of purified pmEVs. pmEVs are labeled to aid in downstream analyses. Alternatively, tumor-bearing mice or mice with some immune disorder (e.g., systemic lupus erythematosus, experimental autoimmune encephalomyelitis, NASH) may be studied for *in vivo* distribution of pmEVs over a given time-course.

[437] Mice can receive a single dose of the pmEV (e.g., 25-100 μ g) or several doses over a defined time course (25-100 μ g). Alternatively, pmEVs dosages may be administered based on particle count (e.g., 7×10^8 to 6×10^{11} particles). Mice are housed under specific pathogen-free conditions following approved protocols. Alternatively, mice may be bred and maintained under sterile, germ-free conditions. Blood, stool, and other tissue samples can be taken at appropriate time points.

[438] The mice are humanely sacrificed at various time points (*i.e.*, hours to days) post administration of the pmEV compositions, and a full necropsy under sterile conditions is performed. Following standard protocols, lymph nodes, adrenal glands, liver, colon, small intestine, cecum, stomach, spleen, kidneys, bladder, pancreas, heart, skin, lungs, brain, and other tissue of interest are harvested and are used directly or snap frozen for further testing. The tissue samples are dissected and homogenized to prepare single-cell suspensions following standard protocols known to one skilled in the art. The number of pmEVs present in the sample is then quantified through flow cytometry. Quantification may also proceed with use of fluorescence microscopy after appropriate processing of whole mouse tissue (Vankelecom H., Fixation and paraffin-embedding of mouse tissues for GFP visualization, *Cold Spring Harb. Protoc.*, 2009). Alternatively, the animals may be analyzed using live-imaging according to the pmEV labeling technique.

[439] Biodistribution may be performed in mouse models of cancer such as but not limited to CT-26 and B16 (see, e.g., Kim et al., Nature Communications vol. 8, no. 626 (2017)) or autoimmunity such as but not limited to EAE and DTH (see, e.g., Turjeman et al., PLoS One 10(7): e0130442 (2015)).

Example 20: Purification and preparation of secreted microbial extracellular vesicles (smEVs) from bacteria

Purification

[440] Secreted microbial extracellular vesicles (smEVs) are purified and prepared from bacterial cultures (e.g., bacteria from Table 2) using methods known to those skilled in the art (S. Bin Park, et al. PLoS ONE. 6(3):e17629 (2011)).

[441] For example, bacterial cultures are centrifuged at 10,000-15,500 x g for 10-40 min at 4°C or room temperature to pellet bacteria. Culture supernatants are then filtered to include material $\leq 0.22 \mu\text{m}$ (for example, via a $0.22 \mu\text{m}$ or $0.45 \mu\text{m}$ filter) and to exclude intact bacterial cells. Filtered supernatants are concentrated using methods that may include, but are not limited to, ammonium sulfate precipitation, ultracentrifugation, or filtration. Briefly, for ammonium sulfate precipitation, 1.5-3 M ammonium sulfate is added to filtered supernatant slowly, while stirring at 4°C. Precipitations are incubated at 4°C for 8-48 hours and then centrifuged at 11,000 x g for 20-40 min at 4°C. The pellets contain smEVs and other debris. Briefly, using ultracentrifugation, filtered supernatants are centrifuged at 100,000-200,000 x g for 1-16 hours at 4°C. The pellet of this centrifugation contains smEVs and other debris. Briefly, using a filtration technique, using an Amicon Ultra spin filter or by tangential flow filtration, supernatants are filtered so as to retain species of molecular weight $> 50, 100, 300, \text{ or } 500 \text{ kDa}$.

[442] Alternatively, smEVs are obtained from bacterial cultures continuously during growth, or at selected time points during growth, by connecting a bioreactor to an alternating tangential flow (ATF) system (e.g., XCell ATF from Repligen) according to manufacturer's instructions. The ATF system retains intact cells ($> 0.22 \mu\text{m}$) in the bioreactor, and allows smaller components (e.g., smEVs, free proteins) to pass through a filter for collection. For example, the system may be configured so that the $< 0.22 \mu\text{m}$ filtrate is then passed through a second filter of 100 kDa, allowing species such as smEVs between $0.22 \mu\text{m}$ and 100 kDa to be collected, and species smaller than 100 kDa to be pumped back into the bioreactor. Alternatively, the system may be configured to allow for medium in the bioreactor to be replenished and/or modified during growth of the culture. smEVs collected by this method may be further purified and/or concentrated by ultracentrifugation or filtration as described above for filtered supernatants.

[443] smEVs obtained by methods described above may be further purified by gradient ultracentrifugation, using methods that may include, but are not limited to, use of a

sucrose gradient or Optiprep gradient. Briefly, using a sucrose gradient method, if ammonium sulfate precipitation or ultracentrifugation were used to concentrate the filtered supernatants, pellets are resuspended in 60% sucrose, 30 mM Tris, pH 8.0. If filtration was used to concentrate the filtered supernatant, the concentrate is buffer exchanged into 60% sucrose, 30 mM Tris, pH 8.0, using an Amicon Ultra column. Samples are applied to a 35-60% discontinuous sucrose gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C. Briefly, using an Optiprep gradient method, if ammonium sulfate precipitation or ultracentrifugation were used to concentrate the filtered supernatants, pellets are resuspended in 45% Optiprep in PBS. If filtration was used to concentrate the filtered supernatant, the concentrate is diluted using 60% Optiprep to a final concentration of 45% Optiprep. Samples are applied to a 0-45% discontinuous sucrose gradient and centrifuged at 200,000 x g for 3-24 hours at 4°C. Alternatively, high resolution density gradient fractionation could be used to separate smEVs based on density.

Preparation

[444] To confirm sterility and isolation of the smEV preparations, smEVs are serially diluted onto agar medium used for routine culture of the bacteria being tested and incubated using routine conditions. Non-sterile preparations are passed through a 0.22 μ m filter to exclude intact cells. To further increase purity, isolated smEVs may be DNase or proteinase K treated.

[445] Alternatively, for preparation of smEVs used for *in vivo* injections, purified smEVs are processed as described previously (G. Norheim, et al. *PLoS ONE*. 10(9): e0134353 (2015)). Briefly, after sucrose gradient centrifugation, bands containing smEVs are resuspended to a final concentration of 50 μ g/mL in a solution containing 3% sucrose or other solution suitable for *in vivo* injection known to one skilled in the art. This solution may also contain adjuvant, for example aluminum hydroxide at a concentration of 0-0.5% (w/v).

[446] To make samples compatible with further testing (e.g., to remove sucrose prior to TEM imaging or *in vitro* assays), samples are buffer exchanged into PBS or 30 mM Tris, pH 8.0 using filtration (e.g., Amicon Ultra columns), dialysis, or ultracentrifugation (following 15-fold or greater dilution in PBS, 200,000 x g, 1-3 hours, 4°C) and resuspension in PBS.

[447] For all of these studies, smEVs may be heated, irradiated, and/or lyophilized prior to administration as described above.

Example 21: Manipulating bacteria through stress to produce various amounts of smEVs and/or to vary content of smEVs

[448] Stress, and in particular envelope stress, has been shown to increase production of smEVs by some bacterial strains (I. MacDonald, M. Kuehn, *J Bacteriol* 195(13): doi: 10/1128/JB.02267-12). In order to vary production of smEVs by bacteria, bacteria are stressed using various methods.

[449] Bacteria may be subjected to single stressors or stressors in combination. The effects of different stressors on different bacteria is determined empirically by varying the stress condition and determining the IC50 value (the conditions required to inhibit cell growth by 50%). smEV purification, quantification, and characterization occurs. smEV production is quantified (1) in complex samples of bacteria and smEVs by nanoparticle tracking analysis (NTA) or transmission electron microscopy (TEM); or (2) following smEV purification by NTA, lipid quantification, or protein quantification. smEV content is assessed following purification by methods described above.

[450] *Antibiotic Stress*

[451] Bacteria are cultivated under standard growth conditions with the addition of sublethal concentrations of antibiotics. This may include 0.1-1 µg/mL chloramphenicol, or 0.1-0.3 µg/mL gentamicin, or similar concentrations of other antibiotics (e.g., ampicillin, polymyxin B). Host antimicrobial products such as lysozyme, defensins, and Reg proteins may be used in place of antibiotics. Bacterially-produced antimicrobial peptides, including bacteriocins and microcins may also be used.

[452] *Temperature Stress*

[453] Bacteria are cultivated under standard growth conditions, but at higher or lower temperatures than are typical for their growth. Alternatively, bacteria are grown under standard conditions, and then subjected to cold shock or heat shock by incubation for a short period of time at low or high temperatures respectively. For example, bacteria grown at 37°C are incubated for 1 hour at 4°C-18°C for cold shock or 42°C-50°C for heat shock.

[454] *Starvation and nutrient limitation*

[455] To induce nutritional stress, bacteria are cultivated under conditions where one or more nutrients are limited. Bacteria may be subjected to nutritional stress throughout growth or shifted from a rich medium to a poor medium. Some examples of media components that are limited are carbon, nitrogen, iron, and sulfur. An example medium is M9 minimal medium (Sigma-Aldrich), which contains low glucose as the sole carbon

source. Media components are also manipulated by the addition of chelators such as EDTA and deferoxamine.

[456] *Saturation*

[457] Bacteria are grown to saturation and incubated past the saturation point for various periods of time. Alternatively, conditioned media is used to mimic saturating environments during exponential growth. Conditioned media is prepared by removing intact cells from saturated cultures by centrifugation and filtration, and conditioned media may be further treated to concentrate or remove specific components.

[458] *Salt Stress*

[459] Bacteria are cultivated in or exposed for brief periods to medium containing NaCl, bile salts, or other salts.

[460] *UV Stress*

[461] UV stress is achieved by cultivating bacteria under a UV lamp or by exposing bacteria to UV using an instrument such as a Stratalinker (Agilent). UV may be administered throughout the entire cultivation period, in short bursts, or for a single defined period following growth.

[462] *Reactive Oxygen Stress*

[463] Bacteria are cultivated in the presence of sublethal concentrations of hydrogen peroxide (250-1,000 μ M) to induce stress in the form of reactive oxygen species. Anaerobic bacteria are cultivated in or exposed to concentrations of oxygen that are toxic to them.

[464] *Detergent stress*

[465] Bacteria are cultivated in or exposed to detergent, such as sodium dodecyl sulfate (SDS) or deoxycholate.

[466] *pH stress*

[467] Bacteria are cultivated in or exposed for limited times to media of different pH.

Example 22: Preparation of smEV-free bacteria

[468] Bacterial samples containing minimal amounts of smEVs are prepared. smEV production is quantified (1) in complex samples of bacteria and extracellular components by NTA or TEM; or (2) following smEV purification from bacterial samples, by NTA, lipid quantification, or protein quantification.

[469] a. Centrifugation and washing: Bacterial cultures are centrifuged at 11,000 x g to separate intact cells from supernatant (including free proteins and vesicles). The pellet is washed with buffer, such as PBS, and stored in a stable way (e.g., mixed with glycerol, flash frozen, and stored at -80°C).

[470] b. ATF: Bacteria and smEVs are separated by connection of a bioreactor to an ATF system. smEV-free bacteria are retained within the bioreactor, and may be further separated from residual smEVs by centrifugation and washing, as described above.

[471] c. Bacteria are grown under conditions that are found to limit production of smEVs. Conditions that may be varied.

Example 23: A colorectal carcinoma model

[472] To study the efficacy of smEVs in a tumor model, one of many cancer cell lines may be used according to rodent tumor models known in the art.

[473] For example, female 6-8 week old Balb/c mice are obtained from Taconic (Germantown, NY) or other vendor. 100,000 CT-26 colorectal tumor cells (ATCC CRL-2638) are resuspended in sterile PBS and inoculated in the presence of 50% Matrigel. CT-26 tumor cells are subcutaneously injected into one hind flank of each mouse. When tumor volumes reach an average of 100mm³ (approximately 10-12 days following tumor cell inoculation), animals are distributed into various treatment groups (e.g., Vehicle; smEVs, with or without anti-PD-1 antibody). Antibodies are administered intraperitoneally (i.p.) at 200 µg/mouse (100 µl final volume) every four days, starting on day 1, for a total of 3 times (Q4Dx3), and smEVs are administered orally or intravenously and at varied doses and varied times. For example, smEVs (5 µg) are intravenously (i.v.) injected every third day, starting on day 1 for a total of 4 times (Q3Dx4) and mice are assessed for tumor growth. Some mice may be intravenously injected with smEVs at 10, 15, or 20 µg smEVs/mouse. Other mice may receive 25, 50, or 100 mg of smEVs per mouse. Alternatively, some mice receive between 7.0e+09 to 3.0e+12 smEV particles per dose.

[474] Alternatively, when tumor volumes reach an average of 100mm³ (approximately 10-12 days following tumor cell inoculation), animals are distributed into the following groups: 1) Vehicle; 2) smEVs; and 3) anti-PD-1 antibody. Antibodies are administered intraperitoneally (i.p.) at 200µg/mouse (100ul final volume) every four days, starting on day 1, and smEVs are administered intraperitoneally (i.p.) daily, starting on day 1 until the conclusion of the study.

[475] When tumor volumes reached an average of 100mm³ (approximately 10-12 days following tumor cell inoculation), animals were distributed into the following groups: 1) Vehicle; 2) anti-PD-1 antibody; and 3) smEV (7.0 e+10 particle count). Antibodies were administered intraperitoneally (i.p.) at 200 µg/mouse (100 µl final volume) every four days, starting on day 1, and smEVs were intravenously (i.v.) injected daily, starting on day 1 until the conclusion of the study and tumors measured for growth. At day 11, the smEV group exhibited tumor growth inhibition that was significantly better than that seen in the anti-PD-1 group. Welch's test is performed for treatment vs. vehicle. In a study looking at dose-response of smEVs, the highest dose of smEVs demonstrated the greatest efficacy, although in a study with smEVs, higher doses do not necessarily correspond to greater efficacy.

Example 24: Administering smEV compositions to treat mouse tumor models

[476] As described in Example 2 a mouse model of cancer is generated by subcutaneously injecting a tumor cell line or patient-derived tumor sample and allowing it to engraft into healthy mice. The methods provided herein may be performed using one of several different tumor cell lines including, but not limited to: B16-F10 or B16-F10-SIY cells as an orthotopic model of melanoma, Panc02 cells as an orthotopic model of pancreatic cancer (Maletzki et al., 2008, Gut 57:483-491), LLC1 cells as an orthotopic model of lung cancer, and RM-1 as an orthotopic model of prostate cancer. As an example, but without limitation, methods for studying the efficacy of smEVs in the B16-F10 model are provided in depth herein.

[477] A syngeneic mouse model of spontaneous melanoma with a very high metastatic frequency is used to test the ability of bacteria to reduce tumor growth and the spread of metastases. The smEVs chosen for this assay are compositions that may display enhanced activation of immune cell subsets and stimulate enhanced killing of tumor cells *in vitro*. The mouse melanoma cell line B16-F10 is obtained from ATCC. The cells are cultured in vitro as a monolayer in RPMI medium, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ in air. The exponentially growing tumor cells are harvested by trypsinization, washed three times with cold 1x PBS, and a suspension of 5E6 cells/ml is prepared for administration. Female C57BL/6 mice are used for this experiment. The mice are 6-8 weeks old and weigh approximately 16-20 g. For tumor development, each mouse is injected SC into the flank with 100 µl of the B16-F10 cell suspension. The mice are anesthetized by ketamine and

xylazine prior to the cell transplantation. The animals used in the experiment may be started on an antibiotic treatment via instillation of a cocktail of kanamycin (0.4 mg/ml), gentamicin, (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml) and vancomycin (0.045 mg/ml) in the drinking water from day 2 to 5 and an intraperitoneal injection of clindamycin (10 mg/kg) on day 7 after tumor injection.

[478] The size of the primary flank tumor is measured with a caliper every 2-3 days and the tumor volume is calculated using the following formula: tumor volume = the tumor width $\times$ tumor length $\times$ 0.5. After the primary tumor reaches approximately 100 mm³, the animals are sorted into several groups based on their body weight. The mice are then randomly taken from each group and assigned to a treatment group. smEV compositions are prepared as previously described. The mice are orally inoculated by gavage with approximately 7.0e+09 to 3.0e+12 smEV particles. Alternatively, smEVs are administered intravenously. Mice receive smEVs daily, weekly, bi-weekly, monthly, bi-monthly, or on any other dosing schedule throughout the treatment period. Mice may be IV injected with smEVs in the tail vein, or directly injected into the tumor. Mice can be injected with smEVs, with or without live bacteria, and/or smEVs with or without inactivated/weakened or killed bacteria. Mice can be injected or orally gavaged weekly or once a month. Mice may receive combinations of purified smEVs and live bacteria to maximize tumor-killing potential. All mice are housed under specific pathogen-free conditions following approved protocols. Tumor size, mouse weight, and body temperature are monitored every 3-4 days and the mice are humanely sacrificed 6 weeks after the B16-F10 mouse melanoma cell injection or when the volume of the primary tumor reaches 1000 mm³. Blood draws are taken weekly and a full necropsy under sterile conditions is performed at the termination of the protocol.

[479] Cancer cells can be easily visualized in the mouse B16-F10 melanoma model due to their melanin production. Following standard protocols, tissue samples from lymph nodes and organs from the neck and chest region are collected and the presence of micro- and macro-metastases is analyzed using the following classification rule. An organ is classified as positive for metastasis if at least two micro-metastatic and one macro-metastatic lesion per lymph node or organ are found. Micro-metastases are detected by staining the paraffin-embedded lymphoid tissue sections with hematoxylin-eosin following standard protocols known to one skilled in the art. The total number of metastases is correlated to the volume of the primary tumor and it is found that the tumor volume correlates significantly with tumor growth time and the number of macro- and micro-metastases in lymph nodes and

visceral organs and also with the sum of all observed metastases. Twenty-five different metastatic sites are identified as previously described (Bobek V., et al., Syngeneic lymph-node-targeting model of green fluorescent protein-expressing Lewis lung carcinoma, Clin. Exp. Metastasis, 2004;21(8):705-8).

[480] The tumor tissue samples are further analyzed for tumor infiltrating lymphocytes. The CD8⁺ cytotoxic T cells can be isolated by FACS and can then be further analyzed using customized p/MHC class I microarrays to reveal their antigen specificity (see e.g., Deviren G., et al., Detection of antigen-specific T cells on p/MHC microarrays, J. Mol. Recognit., 2007 Jan-Feb;20(1):32-8). CD4⁺ T cells can be analyzed using customized p/MHC class II microarrays.

[481] At various timepoints, mice are sacrificed and tumors, lymph nodes, or other tissues may be removed for *ex vivo* flow cytometric analysis using methods known in the art. For example, tumors are dissociated using a Miltenyi tumor dissociation enzyme cocktail according to the manufacturer's instructions. Tumor weights are recorded and tumors are chopped then placed in 15ml tubes containing the enzyme cocktail and placed on ice. Samples are then placed on a gentle shaker at 37°C for 45 minutes and quenched with up to 15ml complete RPMI. Each cell suspension is strained through a 70µm filter into a 50ml falcon tube and centrifuged at 1000 rpm for 10 minutes. Cells are resuspended in FACS buffer and washed to remove remaining debris. If necessary, samples are strained again through a second 70µm filter into a new tube. Cells are stained for analysis by flow cytometry using techniques known in the art. Staining antibodies can include anti-CD11c (dendritic cells), anti-CD80, anti-CD86, anti-CD40, anti-MHCII, anti-CD8a, anti-CD4, and anti-CD103. Other markers that may be analyzed include pan-immune cell marker CD45, T cell markers (CD3, CD4, CD8, CD25, Foxp3, T-bet, Gata3, Rorγt, Granzyme B, CD69, PD-1, CTLA-4), and macrophage/myeloid markers (CD11b, MHCII, CD206, CD40, CSF1R, PD-L1, Gr-1). In addition to immunophenotyping, serum cytokines can be analyzed including, but not limited to, TNFα, IL-17, IL-13, IL-12p70, IL12p40, IL-10, IL-6, IL-5, IL-4, IL-2, IL-1b, IFNγ, GM-CSF, G-CSF, M-CSF, MIG, IP10, MIP1b, RANTES, and MCP-1. Cytokine analysis may be carried out immune cells obtained from lymph nodes or other tissue, and/or on purified CD45⁺ tumor-infiltrated immune cells obtained *ex vivo*. Finally, immunohistochemistry is carried out on tumor sections to measure T cells, macrophages, dendritic cells, and checkpoint molecule protein expression.

[482] The same experiment is also performed with a mouse model of multiple pulmonary melanoma metastases. The mouse melanoma cell line B16-BL6 is obtained from ATCC and the cells are cultured in vitro as described above. Female C57BL/6 mice are used for this experiment. The mice are 6-8 weeks old and weigh approximately 16-20 g. For tumor development, each mouse is injected into the tail vein with 100 μ l of a 2E6 cells/ml suspension of B16-BL6 cells. The tumor cells that engraft upon IV injection end up in the lungs.

[483] The mice are humanely killed after 9 days. The lungs are weighed and analyzed for the presence of pulmonary nodules on the lung surface. The extracted lungs are bleached with Fekete's solution, which does not bleach the tumor nodules because of the melanin in the B16 cells though a small fraction of the nodules is amelanotic (i.e. white). The number of tumor nodules is carefully counted to determine the tumor burden in the mice. Typically, 200-250 pulmonary nodules are found on the lungs of the control group mice (i.e. PBS gavage).

[484] The percentage tumor burden is calculated for the various treatment groups. Percentage tumor burden is defined as the mean number of pulmonary nodules on the lung surfaces of mice that belong to a treatment group divided by the mean number of pulmonary nodules on the lung surfaces of the control group mice.

[485] The tumor biopsies and blood samples are submitted for metabolic analysis via LCMS techniques or other methods known in the art. Differential levels of amino acids, sugars, lactate, among other metabolites, between test groups demonstrate the ability of the microbial composition to disrupt the tumor metabolic state.

RNA Seq to Determine Mechanism of Action

[486] Dendritic cells are purified from tumors, Peyers patches, and mesenteric lymph nodes. RNAseq analysis is carried out and analyzed according to standard techniques known to one skilled in the art (Z. Hou. *Scientific Reports*. 5(9570):doi:10.1038/srep09570 (2015)). In the analysis, specific attention is placed on innate inflammatory pathway genes including TLRs, CLRs, NLRs, and STING, cytokines, chemokines, antigen processing and presentation pathways, cross presentation, and T cell co-stimulation.

[487] Rather than being sacrificed, some mice may be rechallenged with tumor cell injection into the contralateral flank (or other area) to determine the impact of the immune system's memory response on tumor growth.

Example 25: Administering smEVs to treat mouse tumor models in combination with PD-1 or PD-L1 inhibition

[488] To determine the efficacy of smEVs in tumor mouse models in combination with PD-1 or PD-L1 inhibition, a mouse tumor model may be used as described above.

[489] smEVs are tested for their efficacy in the mouse tumor model, either alone or in combination with whole bacterial cells and with or without anti-PD-1 or anti-PD-L1. smEVs, bacterial cells, and/or anti-PD-1 or anti-PD-L1 are administered at varied time points and at varied doses. For example, on day 10 after tumor injection, or after the tumor volume reaches 100mm³, the mice are treated with smEVs alone or in combination with anti-PD-1 or anti-PD-L1.

[490] Mice may be administered smEVs orally, intravenously, or intratumorally. For example, some mice are intravenously injected with anywhere between 7.0e+09 to 3.0e+12 smEV particles. While some mice receive smEVs through i.v. injection, other mice may receive smEVs through intraperitoneal (i.p.) injection, subcutaneous (s.c.) injection, nasal route administration, oral gavage, or other means of administration. Some mice may receive smEVs every day (e.g., starting on day 1), while others may receive smEVs at alternative intervals (e.g., every other day, or once every three days). Groups of mice may be administered a pharmaceutical composition of the invention comprising a mixture of smEVs and bacterial cells. For example, the composition may comprise smEV particles and whole bacteria in a ratio from 1:1 (smEVs: bacterial cells) to 1-1x10¹²:1 (smEVs: bacterial cells).

[491] Alternatively, some groups of mice may receive between 1x10⁴ and 5x10⁹ bacterial cells in an administration separate from, or comingled with, the smEV administration. As with the smEVs, bacterial cell administration may be varied by route of administration, dose, and schedule. The bacterial cells may be live, dead, or weakened. The bacterial cells may be harvested fresh (or frozen) and administered, or they may be irradiated or heat-killed prior to administration with the smEVs.

[492] Some groups of mice are also injected with effective doses of checkpoint inhibitor. For example, mice receive 100 µg anti-PD-L1 mAB (clone 10f.9g2, BioXCell) or another anti-PD-1 or anti-PD-L1 mAB in 100 µl PBS, and some mice receive vehicle and/or other appropriate control (e.g., control antibody). Mice are injected with mABs 3, 6, and 9 days after the initial injection. To assess whether checkpoint inhibition and smEV immunotherapy have an additive anti-tumor effect, control mice receiving anti-PD-1 or anti-PD-L1 mABs are included to the standard control panel. Primary (tumor size) and secondary

(tumor infiltrating lymphocytes and cytokine analysis) endpoints are assessed, and some groups of mice may be rechallenged with a subsequent tumor cell inoculation to assess the effect of treatment on memory response.

Example 26: Labeling bacterial smEVs

[493] smEVs may be labeled in order to track their biodistribution *in vivo* and to quantify and track cellular localization in various preparations and in assays conducted with mammalian cells. For example, smEVs may be radio-labeled, incubated with dyes, fluorescently labeled, luminescently labeled, or labeled with conjugates containing metals and isotopes of metals.

[494] For example, smEVs may be incubated with dyes conjugated to functional groups such as NHS-ester, click-chemistry groups, streptavidin or biotin. The labeling reaction may occur at a variety of temperatures for minutes or hours, and with or without agitation or rotation. The reaction may then be stopped by adding a reagent such as bovine serum albumin (BSA), or similar agent, depending on the protocol, and free or unbound dye molecule removed by ultra-centrifugation, filtration, centrifugal filtration, column affinity purification or dialysis. Additional washing steps involving wash buffers and vortexing or agitation may be employed to ensure complete removal of free dyes molecules such as described in Su Chul Jang et al, *Small*. 11, No.4, 456-461(2017).

[495] Fluorescently labeled smEVs are detected in cells or organs, or in *in vitro* and/or *ex vivo* samples by confocal microscopy, nanoparticle tracking analysis, flow cytometry, fluorescence activated cell sorting (FACs) or fluorescent imaging system such as the Odyssey CLx LICOR (see e.g., Wiklander et al. 2015. *J. Extracellular Vesicles*. 4:10.3402/jev.v4.26316). Additionally, fluorescently labeled smEVs are detected in whole animals and/or dissected organs and tissues using an instrument such as the IVIS spectrum CT (Perkin Elmer) or Pearl Imager, as in H-I. Choi, et al. *Experimental & Molecular Medicine*. 49: e330 (2017).

[496] smEVs may be labeled with conjugates containing metals and isotopes of metals using the protocols described above. Metal-conjugated smEVs may be administered *in vivo* to animals. Cells may then be harvested from organs at various time-points, and analyzed *ex vivo*. Alternatively, cells derived from animals, humans, or immortalized cell lines may be treated with metal-labelled smEVs *in vitro* and cells subsequently labelled with metal-conjugated antibodies and phenotyped using a Cytometry by Time of Flight (CyTOF)

instrument such as the Helios CyTOF (Fluidigm) or imaged and analyzed using an Imaging Mass Cytometry instrument such as the Hyperion Imaging System (Fluidigm). Additionally, smEVs may be labelled with a radioisotope to track the smEVs biodistribution (see, e.g., Miller et al., *Nanoscale*, 2014 May 7;6(9):4928-35).

Example 27: Transmission electron microscopy to visualize purified bacterial smEVs

[497] smEVs are purified from bacteria batch cultures. Transmission electron microscopy (TEM) may be used to visualize purified bacterial smEVs (S. Bin Park, et al. *PLoS ONE*, 6(3):e17629 (2011). smEVs are mounted onto 300- or 400-mesh-size carbon-coated copper grids (Electron Microscopy Sciences, USA) for 2 minutes and washed with deionized water. smEVs are negatively stained using 2% (w/v) uranyl acetate for 20 sec – 1 min. Copper grids are washed with sterile water and dried. Images are acquired using a transmission electron microscope with 100-120 kV acceleration voltage. Stained smEVs appear between 20-600 nm in diameter and are electron dense. 10-50 fields on each grid are screened.

Example 28: Profiling smEV composition and content

[498] smEVs may be characterized by any one of various methods including, but not limited to, NanoSight characterization, SDS-PAGE gel electrophoresis, Western blot, ELISA, liquid chromatography-mass spectrometry and mass spectrometry, dynamic light scattering, lipid levels, total protein, lipid to protein ratios, nucleic acid analysis and/or zeta potential.

NanoSight Characterization of smEVs

[499] Nanoparticle tracking analysis (NTA) is used to characterize the size distribution of purified smEVs. Purified smEV preparations are run on a NanoSight machine (Malvern Instruments) to assess smEV size and concentration.

SDS-PAGE Gel Electrophoresis

[500] To identify the protein components of purified smEVs, samples are run on a gel, for example a Bolt Bis-Tris Plus 4-12% gel (Thermo-Fisher Scientific), using standard techniques. Samples are boiled in 1x SDS sample buffer for 10 minutes, cooled to 4°C, and then centrifuged at 16,000 x g for 1 min. Samples are then run on a SDS-PAGE gel and stained using one of several standard techniques (e.g., Silver staining, Coomassie Blue, Gel Code Blue) for visualization of bands.

Western blot analysis

[501] To identify and quantify specific protein components of purified smEVs, smEV proteins are separated by SDS-PAGE as described above and subjected to Western blot analysis (Cvijetkovic et al., *Sci. Rep.* 6, 36338 (2016)) and are quantified via ELISA. *smEV proteomics and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Mass Spectrometry (MS)*

[502] Proteins present in smEVs are identified and quantified by Mass Spectrometry techniques. smEV proteins may be prepared for LC-MS/MS using standard techniques including protein reduction using dithiotreitol solution (DTT) and protein digestion using enzymes such as LysC and trypsin as described in Erickson et al, 2017 (Molecular Cell, VOLUME 65, ISSUE 2, P361-370, JANUARY 19, 2017). Alternatively, peptides are prepared as described by Liu et al. 2010 (JOURNAL OF BACTERIOLOGY, June 2010, p. 2852–2860 Vol. 192, No. 11), Kieselbach and Oscarsson 2017 (Data Brief. 2017 Feb; 10: 426–431.), Vildhede et al, 2018 (Drug Metabolism and Disposition February 8, 2018). Following digestion, peptide preparations are run directly on liquid chromatography and mass spectrometry devices for protein identification within a single sample. For relative quantitation of proteins between samples, peptide digests from different samples are labeled with isobaric tags using the iTRAQ Reagent-8plex Multiplex Kit (Applied Biosystems, Foster City, CA) or TMT 10plex and 11plex Label Reagents (Thermo Fischer Scientific, San Jose, CA, USA). Each peptide digest is labeled with a different isobaric tag and then the labeled digests are combined into one sample mixture. The combined peptide mixture is analyzed by LC-MS/MS for both identification and quantification. A database search is performed using the LC-MS/MS data to identify the labeled peptides and the corresponding proteins. In the case of isobaric labeling, the fragmentation of the attached tag generates a low molecular mass reporter ion that is used to obtain a relative quantitation of the peptides and proteins present in each smEV.

[503] Additionally, metabolic content is ascertained using liquid chromatography techniques combined with mass spectrometry. A variety of techniques exist to determine metabolomic content of various samples and are known to one skilled in the art involving solvent extraction, chromatographic separation and a variety of ionization techniques coupled to mass determination (Roberts et al 2012 Targeted Metabolomics. Curr Protoc Mol Biol. 30: 1-24; Dettmer et al 2007, Mass spectrometry-based metabolomics. Mass Spectrom Rev. 26(1):51-78). As a non-limiting example, a LC-MS system includes a 4000 QTRAP

triple quadrupole mass spectrometer (AB SCIEX) combined with 1100 Series pump (Agilent) and an HTS PAL autosampler (Leap Technologies). Media samples or other complex metabolic mixtures (~10 µL) are extracted using nine volumes of 74.9:24.9:0.2 (v/v/v) acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (valine-d8, Isotec; and phenylalanine-d8, Cambridge Isotope Laboratories). Standards may be adjusted or modified depending on the metabolites of interest. The samples are centrifuged (10 minutes, 9,000g, 4 °C), and the supernatants (10 µL) are submitted to LCMS by injecting the solution onto the HILIC column (150 × 2.1 mm, 3 µm particle size). The column is eluted by flowing a 5% mobile phase [10mM ammonium formate, 0.1% formic acid in water] for 1 minute at a rate of 250uL/minute followed by a linear gradient over 10 minutes to a solution of 40% mobile phase [acetonitrile with 0.1% formic acid]. The ion spray voltage is set to 4.5 kV and the source temperature is 450 °C.

[504] The data are analyzed using commercially available software like Multiquant 1.2 from AB SCIEX for mass spectrum peak integration. Peaks of interest should be manually curated and compared to standards to confirm the identity of the peak. Quantitation with appropriate standards is performed to determine the number of metabolites present in the initial media, after bacterial conditioning and after tumor cell growth. A non-targeted metabolomics approach may also be used using metabolite databases, such as but not limited to the NIST database, for peak identification.

[505] *Dynamic light scattering (DLS)*

[506] DLS measurements, including the distribution of particles of different sizes in different smEV preparations are taken using instruments such as the DynaPro NanoStar (Wyatt Technology) and the Zetasizer Nano ZS (Malvern Instruments).

Lipid Levels

[507] Lipid levels are quantified using FM4-64 (Life Technologies), by methods similar to those described by A.J. McBroom *et al. J Bacteriol* 188:5385-5392. and A. Frias, *et al. Microb Ecol.* 59:476-486 (2010). Samples are incubated with FM4-64 (3.3 µg/mL in PBS for 10 minutes at 37°C in the dark). After excitation at 515 nm, emission at 635 nm is measured using a Spectramax M5 plate reader (Molecular Devices). Absolute concentrations are determined by comparison of unknown samples to standards (such as palmitoyloleoylphosphatidylglycerol (POPG) vesicles) of known concentrations. Lipidomics can be used to identify the lipids present in the smEVs.

Total Protein

[508] Protein levels are quantified by standard assays such as the Bradford and BCA assays. The Bradford assays are run using Quick Start Bradford 1x Dye Reagent (Bio-Rad), according to manufacturer's protocols. BCA assays are run using the Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific). Absolute concentrations are determined by comparison to a standard curve generated from BSA of known concentrations. Alternatively, protein concentration can be calculated using the Beer-Lambert equation using the sample absorbance at 280nm (A₂₈₀) as measured on a Nanodrop spectrophotometer (Thermo-Fisher Scientific). In addition, proteomics may be used to identify proteins in the sample.

Lipid:Protein Ratios

[509] Lipid:protein ratios are generated by dividing lipid concentrations by protein concentrations. These provide a measure of the purity of vesicles as compared to free protein in each preparation.

Nucleic acid analysis

[510] Nucleic acids are extracted from smEVs and quantified using a Qubit fluorometer. Size distribution is assessed using a BioAnalyzer and the material is sequenced.

Zeta Potential

[511] The zeta potential of different preparations are measured using instruments such as the Zetasizer ZS (Malvern Instruments).

Example 29: In vitro screening of smEVs for enhanced activation of dendritic cells

[512] *In vitro* immune responses are thought to simulate mechanisms by which immune responses are induced *in vivo*, e.g., as in response to a cancer microenvironment. Briefly, PBMCs are isolated from heparinized venous blood from healthy donors by gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway), or from mouse spleens or bone marrow using the magnetic bead-based Human Blood Dendritic cell isolation kit (Miltenyi Biotech, Cambridge, MA). Using anti-human CD14 mAb, the monocytes are purified by MoFlo and cultured in cRPMI at a cell density of 5e5 cells/ml in a 96-well plate (Costar Corp) for 7 days at 37°C. For maturation of dendritic cells, the culture is stimulated with 0.2 ng/mL IL-4 and 1000 U/ml GM-CSF at 37°C for one week. Alternatively, maturation is achieved through incubation with recombinant GM-CSF for a week, or using other methods known in the art. Mouse DCs can be harvested directly from spleens using bead enrichment or differentiated from hematopoietic stem cells. Briefly, bone marrow may be obtained from

the femurs of mice. Cells are recovered and red blood cells lysed. Stem cells are cultured in cell culture medium in 20ng/ml mouse GM-CSF for 4 days. Additional medium containing 20ng/ml mouse GM-CSF is added. On day 6 the medium and non-adherent cells are removed and replaced with fresh cell culture medium containing 20ng/ml GM-CSF. A final addition of cell culture medium with 20ng/ml GM-CSF is added on day 7. On day 10, non-adherent cells are harvested and seeded into cell culture plates overnight and stimulated as required. Dendritic cells are then treated with various doses of smEVs with or without antibiotics. For example, 25-75 ug/mL smEVs for 24 hours with antibiotics. smEV compositions tested may include smEVs from a single bacterial species or strain, or a mixture of smEVs from one or more genus, 1 or more species, or 1 or more strains (e.g., one or more strains within one species). PBS is included as a negative control and LPS, anti-CD40 antibodies, and/or smEVs are used as positive controls. Following incubation, DCs are stained with anti CD11b, CD11c, CD103, CD8a, CD40, CD80, CD83, CD86, MHC I and MHC II, and analyzed by flow cytometry. DCs that are significantly increased in CD40, CD80, CD83, and CD86 as compared to negative controls are considered to be activated by the associated bacterial smEV composition. These experiments are repeated three times at minimum.

[513] To screen for the ability of smEV-activated epithelial cells to stimulate DCs, the above protocol is followed with the addition of a 24-hour epithelial cell smEV co-culture prior to incubation with DCs. Epithelial cells are washed after incubation with smEVs and are then co-cultured with DCs in an absence of smEVs for 24 hours before being processed as above. Epithelial cell lines may include Int407, HEL293, HT29, T84 and CACO2.

[514] As an additional measure of DC activation, 100 µl of culture supernatant is removed from wells following 24-hour incubation of DCs with smEVs or smEV-treated epithelial cells and is analyzed for secreted cytokines, chemokines, and growth factors using the multiplexed Luminex Magpix. Kit (EMD Millipore, Darmstadt, Germany). Briefly, the wells are pre-wet with buffer, and 25 µl of 1x antibody-coated magnetic beads are added and 2x 200 µl of wash buffer are performed in every well using the magnet. 50 µl of Incubation buffer, 50 µl of diluent and 50 µl of samples are added and mixed via shaking for 2hrs at room temperature in the dark. The beads are then washed twice with 200 µl wash buffer. 100 µl of 1X biotinylated detector antibody is added and the suspension is incubated for 1 hour with shaking in the dark. Two, 200 µl washes are then performed with wash buffer. 100 µl of 1x SAV-RPE reagent is added to each well and is incubated for 30 min at RT in the dark.

Three 200 µl washes are performed and 125 µl of wash buffer is added with 2-3 min shaking occurs. The wells are then submitted for analysis in the Luminex xMAP system.

[515] Standards allow for careful quantitation of the cytokines including GM-CSF, IFN-γ, IFN-α, IFN-β, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12 (p40/p70), IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IP-10, KC, MCP-1, MIG, MIP1α, TNFα, and VEGF. These cytokines are assessed in samples of both mouse and human origin. Increases in these cytokines in the bacterial treated samples indicate enhanced production of proteins and cytokines from the host. Other variations on this assay examining specific cell types ability to release cytokines are assessed by acquiring these cells through sorting methods and are recognized by one of ordinary skill in the art. Furthermore, cytokine mRNA is also assessed to address cytokine release in response to an smEV composition.

[516] This DC stimulation protocol may be repeated using combinations of purified smEVs and live bacterial strains to maximize immune stimulation potential.

Example 30: In vitro screening of smEVs for enhanced activation of CD8+ T cell killing when incubated with tumor cells

[517] *In vitro* methods for screening smEVs that can activate CD8+ T cell killing of tumor cells are described. Briefly, DCs are isolated from human PBMCs or mouse spleens, using techniques known in the art, and incubated *in vitro* with single-strain smEVs, mixtures of smEVs, and/or appropriate controls. In addition, CD8+ T cells are obtained from human PBMCs or mouse spleens using techniques known in the art, for example the magnetic bead-based Mouse CD8α+ T Cell Isolation Kit and the magnetic bead-based Human CD8+ T Cell Isolation Kit (both from Miltenyi Biotech, Cambridge, MA). After incubation of DCs with smEVs for some time (e.g., for 24-hours), or incubation of DCs with smEV-stimulated epithelial cells, smEVs are removed from the cell culture with PBS washes and 100ul of fresh media with antibiotics is added to each well, and 200,000 T cells are added to each experimental well in the 96-well plate. Anti-CD3 antibody is added at a final concentration of 2ug/ml. Co-cultures are then allowed to incubate at 37°C for 96 hours under normal oxygen conditions.

[518] For example, approximately 72 hours into the coculture incubation, tumor cells are plated for use in the assay using techniques known in the art. For example, 50,000 tumor cells/well are plated per well in new 96-well plates. Mouse tumor cell lines used may include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to

donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. After completion of the 96-hour co-culture, 100 μ l of the CD8⁺ T cell and DC mixture is transferred to wells containing tumor cells. Plates are incubated for 24 hours at 37°C under normal oxygen conditions. Staurosporine may be used as negative control to account for cell death.

[519] Following this incubation, flow cytometry is used to measure tumor cell death and characterize immune cell phenotype. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well. Cytotoxic CD8⁺ T cell phenotype may be characterized by the following methods: a) concentration of supernatant granzyme B, IFN γ and TNF α in the culture supernatant as described below, b) CD8⁺ T cell surface expression of activation markers such as DC69, CD25, CD154, PD-1, gamma/delta TCR, Foxp3, T-bet, granzyme B, c) intracellular cytokine staining of IFN γ , granzyme B, TNF α in CD8⁺ T cells. CD4⁺ T cell phenotype may also be assessed by intracellular cytokine staining in addition to supernatant cytokine concentration including IFN γ , TNF α , IL-12, IL-4, IL-5, IL-17, IL-10, chemokines etc.

[520] As an additional measure of CD8⁺ T cell activation, 100 μ l of culture supernatant is removed from wells following the 96-hour incubation of T cells with DCs and is analyzed for secreted cytokines, chemokines, and growth factors using the multiplexed Luminex Magpix. Kit (EMD Millipore, Darmstadt, Germany). Briefly, the wells are pre-wet with buffer, and 25 μ l of 1x antibody-coated magnetic beads are added and 2x 200 μ l of wash buffer are performed in every well using the magnet. 50 μ l of Incubation buffer, 50 μ l of diluent and 50 μ l of samples are added and mixed via shaking for 2hrs at room temperature in the dark. The beads are then washed twice with 200 μ l wash buffer. 100 μ l of 1X biotinylated detector antibody is added and the suspension is incubated for 1 hour with shaking in the dark. Two, 200 μ l washes are then performed with wash buffer. 100 μ l of 1x SAV-RPE reagent is added to each well and is incubated for 30 min at RT in the dark. Three 200 μ l washes are performed and 125 μ l of wash buffer is added with 2-3 min shaking occurs. The wells are then submitted for analysis in the Luminex xMAP system.

[521] Standards allow for careful quantitation of the cytokines including GM-CSF, IFN- γ , IFN- α , IFN- β , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12 (p40/p70), IL-17, IL-23, IP-10, KC, MCP-1, MIG, MIP1 α , TNF α , and VEGF. These cytokines are assessed in samples of both mouse and human origin. Increases in these

cytokines in the bacterial treated samples indicate enhanced production of proteins and cytokines from the host. Other variations on this assay examining specific cell types ability to release cytokines are assessed by acquiring these cells through sorting methods and are recognized by one of ordinary skill in the art. Furthermore, cytokine mRNA is also assessed to address cytokine release in response to an smEV composition. These changes in the cells of the host stimulate an immune response similarly to in vivo response in a cancer microenvironment.

[522] This CD8+ T cell stimulation protocol may be repeated using combinations of purified smEVs and live bacterial strains to maximize immune stimulation potential.

Example 31: In vitro screening of smEVs for enhanced tumor cell killing by PBMCs

[523] Various methods may be used to screen smEVs for the ability to stimulate PBMCs, which in turn activate CD8+ T cells to kill tumor cells. For example, PBMCs are isolated from heparinized venous blood from healthy human donors by ficoll-paque gradient centrifugation for mouse or human blood, or with Lympholyte Cell Separation Media (Cedarlane Labs, Ontario, Canada) from mouse blood. PBMCs are incubated with single-strain smEVs, mixtures of smEVs, and appropriate controls. In addition, CD8+ T cells are obtained from human PBMCs or mouse spleens. After the 24-hour incubation of PBMCs with smEVs, smEVs are removed from the cells using PBS washes. 100ul of fresh media with antibiotics is added to each well. An appropriate number of T cells (e.g., 200,000 T cells) are added to each experimental well in the 96-well plate. Anti-CD3 antibody is added at a final concentration of 2ug/ml. Co-cultures are then allowed to incubate at 37°C for 96 hours under normal oxygen conditions.

[524] For example, 72 hours into the coculture incubation, 50,000 tumor cells/well are plated per well in new 96-well plates. Mouse tumor cell lines used include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. After completion of the 96-hour co-culture, 100 µl of the CD8+ T cell and PBMC mixture is transferred to wells containing tumor cells. Plates are incubated for 24 hours at 37°C under normal oxygen conditions. Staurosporine is used as negative control to account for cell death.

[525] Following this incubation, flow cytometry is used to measure tumor cell death and characterize immune cell phenotype. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of

dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well. Cytotoxic CD8+ T cell phenotype may be characterized by the following methods: a) concentration of supernatant granzyme B, IFN γ and TNF α in the culture supernatant as described below, b) CD8+ T cell surface expression of activation markers such as DC69, CD25, CD154, PD-1, gamma/delta TCR, Foxp3, T-bet, granzyme B, c) intracellular cytokine staining of IFN γ , granzyme B, TNF α in CD8+ T cells. CD4+ T cell phenotype may also be assessed by intracellular cytokine staining in addition to supernatant cytokine concentration including IFN γ , TNF α , IL-12, IL-4, IL-5, IL-17, IL-10, chemokines etc.

[526] As an additional measure of CD8+ T cell activation, 100 μ l of culture supernatant is removed from wells following the 96-hour incubation of T cells with DCs and is analyzed for secreted cytokines, chemokines, and growth factors using the multiplexed Luminex Magpix. Kit (EMD Millipore, Darmstadt, Germany). Briefly, the wells are pre-wet with buffer, and 25 μ l of 1x antibody-coated magnetic beads are added and 2x 200 μ l of wash buffer are performed in every well using the magnet. 50 μ l of Incubation buffer, 50 μ l of diluent and 50 μ l of samples are added and mixed via shaking for 2hrs at room temperature in the dark. The beads are then washed twice with 200 μ l wash buffer. 100 μ l of 1X biotinylated detector antibody is added and the suspension is incubated for 1 hour with shaking in the dark. Two, 200 μ l washes are then performed with wash buffer. 100 μ l of 1x SAV-RPE reagent is added to each well and is incubated for 30 min at RT in the dark. Three 200 μ l washes are performed and 125 μ l of wash buffer is added with 2-3 min shaking occurs. The wells are then submitted for analysis in the Luminex xMAP system.

[527] Standards allow for careful quantitation of the cytokines including GM-CSF, IFN- γ , IFN- α , IFN- β IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12 (p40/p70), IL-17, IL-23, IP-10, KC, MCP-1, MIG, MIP1 α , TNF α , and VEGF. These cytokines are assessed in samples of both mouse and human origin. Increases in these cytokines in the bacterial treated samples indicate enhanced production of proteins and cytokines from the host. Other variations on this assay examining specific cell types ability to release cytokines are assessed by acquiring these cells through sorting methods and are recognized by one of ordinary skill in the art. Furthermore, cytokine mRNA is also assessed to address cytokine release in response to an smEV composition. These changes in the cells of the host stimulate an immune response similarly to in vivo response in a cancer microenvironment.

[528] This PBMC stimulation protocol may be repeated using combinations of purified smEVs with or without combinations of live, dead, or inactivated/weakened bacterial strains to maximize immune stimulation potential.

Example 32: In vitro detection of smEVs in antigen-presenting cells

[529] Dendritic cells in the lamina propria constantly sample live bacteria, dead bacteria, and microbial products in the gut lumen by extending their dendrites across the gut epithelium, which is one way that smEVs produced by bacteria in the intestinal lumen may directly stimulate dendritic cells. The following methods represent a way to assess the differential uptake of smEVs by antigen-presenting cells. Optionally, these methods may be applied to assess immunomodulatory behavior of smEVs administered to a patient.

[530] Dendritic cells (DCs) are isolated from human or mouse bone marrow, blood, or spleens according to standard methods or kit protocols (e.g., Inaba K, Swiggard WJ, Steinman RM, Romani N, Schuler G, 2001. Isolation of dendritic cells. Current Protocols in Immunology. Chapter 3:Unit3.7).

[531] To evaluate smEV entrance into and/or presence in DCs, 250,000 DCs are seeded on a round cover slip in complete RPMI-1640 medium and are then incubated with smEVs from single bacterial strains or combinations smEVs at various ratios. Purified smEVs may be labeled with fluorochromes or fluorescent proteins. After incubation for various timepoints (e.g., 1 hour, 2 hours), the cells are washed twice with ice-cold PBS and detached from the plate using trypsin. Cells are either allowed to remain intact or are lysed. Samples are then processed for flow cytometry. Total internalized smEVs are quantified from lysed samples, and percentage of cells that uptake smEVs is measured by counting fluorescent cells. The methods described above may also be performed in substantially the same manner using macrophages or epithelial cell lines (obtained from the ATCC) in place of DCs.

Example 33: In vitro screening of smEVs with an enhanced ability to activate NK cell killing when incubated with target cells

[532] To demonstrate the ability of the selected smEV compositions to elicit potent NK cell cytotoxicity to tumor cells, the following *in vitro* assay is used. Briefly, mononuclear cells from heparinized blood are obtained from healthy human donors. Optionally, an expansion step to increase the numbers of NK cells is performed as

previously described (e.g., see Somanschi et al., *J Vis Exp.* 2011;(48):2540). The cells may be adjusted to a concentration of ,cells/ml in RPMI-1640 medium containing 5% human serum. The PMNC cells are then labeled with appropriate antibodies and NK cells are isolated through FACS as CD3-/CD56+ cells and are ready for the subsequent cytotoxicity assay. Alternatively, NK cells are isolated using the autoMACs instrument and NK cell isolation kit following manufacturer's instructions (Miltenyl Biotec).

[533] NK cells are counted and plated in a 96 well format with 20,000 or more cells per well, and incubated with single-strain smEVs, with or without addition of antigen presenting cells (e.g., monocytes derived from the same donor), smEVs from mixtures of bacterial strains, and appropriate controls. After 5-24 hours incubation of NK cells with smEVs, smEVs are removed from cells with PBS washes, NK cells are resuspended in 10 mL fresh media with antibiotics and are added to 96-well plates containing 20,000 target tumor cells/well. Mouse tumor cell lines used include B16.F10, SIY+ B16.F10, and others. Human tumor cell lines are HLA-matched to donor, and can include PANC-1, UNKPC960/961, UNKC, and HELA cell lines. Plates are incubated for 2-24 hours at 37°C under normal oxygen conditions. Staurosporine is used as negative control to account for cell death.

[534] Following this incubation, flow cytometry is used to measure tumor cell death using methods known in the art. Briefly, tumor cells are stained with viability dye. FACS analysis is used to gate specifically on tumor cells and measure the percentage of dead (killed) tumor cells. Data are also displayed as the absolute number of dead tumor cells per well.

[535] This NK cell stimulation protocol may be repeated using combinations of purified smEVs and live bacterial strains to maximize immune stimulation potential.

Example 34: Using in vitro immune activation assays to predict in vivo cancer immunotherapy efficacy of smEV compositions

[536] *In vitro* immune activation assays identify smEVs that are able to stimulate dendritic cells, which in turn activate CD8+ T cell killing. Therefore, the *in vitro* assays described above are used as a predictive screen of a large number of candidate smEVs for potential immunotherapy activity. smEVs that display enhanced stimulation of dendritic cells, enhanced stimulation of CD8+ T cell killing, enhanced stimulation of PBMC killing,

and/or enhanced stimulation of NK cell killing, are preferentially chosen for *in vivo* cancer immunotherapy efficacy studies.

Example 35: Determining the biodistribution of smEVs when delivered orally to mice

[537] Wild-type mice (e.g., C57BL/6 or BALB/c) are orally inoculated with the smEV composition of interest to determine the *in vivo* biodistribution profile of purified smEVs. smEVs are labeled to aide in downstream analyses. Alternatively, tumor-bearing mice or mice with some immune disorder (e.g., systemic lupus erythematosus, experimental autoimmune encephalomyelitis, NASH) may be studied for *in vivo* distribution of smEVs over a given time-course.

[538] Mice can receive a single dose of the smEV (e.g., 25-100 μ g) or several doses over a defined time course (25-100 μ g). Alternatively, smEVs dosages may be administered based on particle count (e.g., $7e+08$ to $6e+11$ particles). Mice are housed under specific pathogen-free conditions following approved protocols. Alternatively, mice may be bred and maintained under sterile, germ-free conditions. Blood, stool, and other tissue samples can be taken at appropriate time points.

[539] The mice are humanely sacrificed at various time points (*i.e.*, hours to days) post administration of the smEV compositions, and a full necropsy under sterile conditions is performed. Following standard protocols, lymph nodes, adrenal glands, liver, colon, small intestine, cecum, stomach, spleen, kidneys, bladder, pancreas, heart, skin, lungs, brain, and other tissue of interest are harvested and are used directly or snap frozen for further testing. The tissue samples are dissected and homogenized to prepare single-cell suspensions following standard protocols known to one skilled in the art. The number of smEVs present in the sample is then quantified through flow cytometry. Quantification may also proceed with use of fluorescence microscopy after appropriate processing of whole mouse tissue (Vankelecom H., Fixation and paraffin-embedding of mouse tissues for GFP visualization, *Cold Spring Harb. Protoc.*, 2009). Alternatively, the animals may be analyzed using live-imaging according to the smEV labeling technique.

[540] Biodistribution may be performed in mouse models of cancer such as but not limited to CT-26 and B16 (see, e.g., Kim et al., *Nature Communications* vol. 8, no. 626 (2017)) or autoimmunity such as but not limited to EAE and DTH (see, e.g., Turjeman et al., *PLoS One* 10(7): e0130442 (20105).

Example 36: Manufacturing conditions

[541] Enriched media is used to grow and prepare the bacteria for *in vitro* and *in vivo* use and, ultimately, for pmEV and smEV preparations. For example, media may contain sugar, yeast extracts, plant-based peptones, buffers, salts, trace elements, surfactants, anti-foaming agents, and vitamins. Composition of complex components such as yeast extracts and peptones may be undefined or partially defined (including approximate concentrations of amino acids, sugars etc.). Microbial metabolism may be dependent on the availability of resources such as carbon and nitrogen. Various sugars or other carbon sources may be tested. Alternatively, media may be prepared and the selected bacterium grown as shown by Saarela *et al.*, *J. Applied Microbiology*, 2005, 99: 1330-1339, which is hereby incorporated by reference. Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of the selected bacterium produced without milk-based ingredients.

[542] At large scale, the media is sterilized. Sterilization may be accomplished by Ultra High Temperature (UHT) processing. The UHT processing is performed at very high temperature for short periods of time. The UHT range may be from 135-180°C. For example, the medium may be sterilized from between 10 to 30 seconds at 135°C.

[543] Inoculum can be prepared in flasks or in smaller bioreactors and growth is monitored. For example, the inoculum size may be between approximately 0.5 and 3% of the total bioreactor volume. Depending on the application and need for material, bioreactor volume can be at least 2L, 10L, 80L, 100L, 250L, 1000L, 2500L, 5000L, 10,000L.

[544] Before the inoculation, the bioreactor is prepared with medium at desired pH, temperature, and oxygen concentration. The initial pH of the culture medium may be different than the process set-point. pH stress may be detrimental at low cell concentration; the initial pH could be between pH 7.5 and the process set-point. For example, pH may be set between 4.5 and 8.0. During the fermentation, the pH can be controlled through the use of sodium hydroxide, potassium hydroxide, or ammonium hydroxide. The temperature may be controlled from 25°C to 45°C, for example at 37°C. Anaerobic conditions are created by reducing the level of oxygen in the culture broth from around 8mg/L to 0mg/L. For example, nitrogen or gas mixtures (N₂, CO₂, and H₂) may be used in order to establish anaerobic conditions. Alternatively, no gases are used and anaerobic conditions are established by cells consuming remaining oxygen from the medium. Depending on strain and inoculum size, the

bioreactor fermentation time can vary. For example, fermentation time can vary from approximately 5 hours to 48 hours.

[545] Reviving microbes from a frozen state may require special considerations. Production medium may stress cells after a thaw; a specific thaw medium may be required to consistently start a seed train from thawed material. The kinetics of transfer or passage of seed material to fresh medium, for the purposes of increasing the seed volume or maintaining the microbial growth state, may be influenced by the current state of the microbes (ex. exponential growth, stationary growth, unstressed, stressed).

[546] Inoculation of the production fermenter(s) can impact growth kinetics and cellular activity. The initial state of the bioreactor system must be optimized to facilitate successful and consistent production. The fraction of seed culture to total medium (e.g., a percentage) has a dramatic impact on growth kinetics. The range may be 1-5% of the fermenter's working volume. The initial pH of the culture medium may be different from the process set-point. pH stress may be detrimental at low cell concentration; the initial pH may be between pH 7.5 and the process set-point. Agitation and gas flow into the system during inoculation may be different from the process set-points. Physical and chemical stresses due to both conditions may be detrimental at low cell concentration.

[547] Process conditions and control settings may influence the kinetics of microbial growth and cellular activity. Shifts in process conditions may change membrane composition, production of metabolites, growth rate, cellular stress, etc. Optimal temperature range for growth may vary with strain. The range may be 20-40 °C. Optimal pH for cell growth and performance of downstream activity may vary with strain. The range may be pH 5-8. Gasses dissolved in the medium may be used by cells for metabolism. Adjusting concentrations of O₂, CO₂, and N₂ throughout the process may be required. Availability of nutrients may shift cellular growth. Microbes may have alternate kinetics when excess nutrients are available.

[548] The state of microbes at the end of a fermentation and during harvesting may impact cell survival and activity. Microbes may be preconditioned shortly before harvest to better prepare them for the physical and chemical stresses involved in separation and downstream processing. A change in temperature (often reducing to 20-5 °C) may reduce cellular metabolism, slowing growth (and/or death) and physiological change when removed from the fermenter. Effectiveness of centrifugal concentration may be influenced by culture pH. Raising pH by 1-2 points can improve effectiveness of concentration but can also be

detrimental to cells. Microbes may be stressed shortly before harvest by increasing the concentration of salts and/or sugars in the medium. Cells stressed in this way may better survive freezing and lyophilization during downstream.

[549] Separation methods and technology may impact how efficiently microbes are separated from the culture medium. Solids may be removed using centrifugation techniques. Effectiveness of centrifugal concentration can be influenced by culture pH or by the use of flocculating agents. Raising pH by 1-2 points may improve effectiveness of concentration but can also be detrimental to cells. Microbes may be stressed shortly before harvest by increasing the concentration of salts and/or sugars in the medium. Cells stressed in this way may better survive freezing and lyophilization during downstream. Additionally, Microbes may also be separated via filtration. Filtration is superior to centrifugation techniques for purification if the cells require excessive g-minutes to successfully centrifuge. Excipients can be added before after separation. Excipients can be added for cryo protection or for protection during lyophilization. Excipients can include, but are not limited to, sucrose, trehalose, or lactose, and these may be alternatively mixed with buffer and anti-oxidants. Prior to lyophilization, droplets of cell pellets mixed with excipients are submerged in liquid nitrogen.

[550] Harvesting can be performed by continuous centrifugation. Product may be resuspended with various excipients to a desired final concentration. Excipients can be added for cryo protection or for protection during lyophilization. Excipients can include, but are not limited to, sucrose, trehalose, or lactose, and these may be alternatively mixed with buffer and anti-oxidants. Prior to lyophilization, droplets of cell pellets mixed with excipients are submerged in liquid nitrogen.

[551] Lyophilization of material, including live bacteria, vesicles, or other bacterial derivative includes a freezing, primary drying, and secondary drying phase. Lyophilization begins with freezing. The product material may or may not be mixed with a lyoprotectant or stabilizer prior to the freezing stage. A product may be frozen prior to the loading of the lyophilizer, or under controlled conditions on the shelf of the lyophilizer. During the next phase, the primary drying phase, ice is removed via sublimation. Here, a vacuum is generated and an appropriate amount of heat is supplied to the material. The ice will sublime while keeping the product temperature below freezing, and below the material's critical temperature (T_c). The temperature of the shelf on which the material is loaded and the chamber vacuum can be manipulated to achieve the desired product

temperature. During the secondary drying phase, product-bound water molecules are removed. Here, the temperature is generally raised higher than in the primary drying phase to break any physico-chemical interactions that have formed between the water molecules and the product material. After the freeze-drying process is complete, the chamber may be filled with an inert gas, such as nitrogen. The product may be sealed within the freeze dryer under dry conditions, in a glass vial or other similar container, preventing exposure to atmospheric water and contaminants.

Example 37: smEV and pmEV preparation

[552] *smEVs:* Downstream processing of smEVs begins immediately following harvest of the bioreactor. Centrifugation at 20,000 g is used to remove the cells from the broth. The resulting supernatant is clarified using 0.22 µm filter. The smEVs are concentrated and washed using tangential flow filtration (TFF) with flat sheet cassettes ultrafiltration (UF) membranes with 100 kDa molecular weight cutoff (MWCO). Diafiltration (DF) is used to washout small molecules and small proteins using 5 volumes of phosphate buffer solution (PBS). The retentate from TFF is spun down in an ultracentrifuge at 200,000 g for 1 hour to form a pellet rich in smEVs called a high-speed pellet (HSP). The pellet is resuspended with minimal PBS and a gradient was prepared with optiprep™ density gradient medium and ultracentrifuged at 200,000 g for 16 hours. Of the resulting fractions, 2 middle bands contain smEVs. The fractions are washed with 15 fold PBS and the smEVs are spun down at 200,000 g for 1 hr to create the fractionated HSP or fHSP. It is subsequently resuspended with minimal PBS, pooled, and analyzed for particles per mL and protein content. Dosing is prepared from the particle / mL count to achieve desired concentration. The smEVs are characterized using a NanoSight NS300 by Malvern Panalytical in scatter mode using the 532 nm laser.

[553] *pmEVs:*

[554] Cell pellets are removed from freezer and placed on ice. Pellet weights are noted.

[555] Cold 100 mM Tris-HCl pH 7.5 is added to the frozen pellets and the pellets are thawed rotating at 4°C.

[556] 10mg/ml DNase stock is added to the thawed pellets to a final concentration of 1mg/mL.

- [557] The pellets are incubated on the inverter for 40 min at RT (room temperature).
- [558] The sample is filtered in a 70um cell strainer before running through the Emulsiflex.
- [559] The samples are lysed using the Emulsiflex with 8 discrete cycles at 22,000psi.
- [560] To remove the cellular debris from the lysed sample, the sample is centrifuged at 12,500 x g, 15 min, 4°C.
- [561] The sample is centrifuged two additional times at 12,500 x g, 15 min, 4°C, each time moving the supernatant to a fresh tube.
- [562] To pellet the membrane proteins, the sample is centrifuged at 120,000 x g, 1 hr, 4°C.
- [563] The pellet is resuspended in 10 mL ice-cold 0.1 M sodium carbonate pH 11. The sample is incubated on the inverter at 4°C for 1 hour.
- [564] The sample is centrifuged at 120,000 x g, 1 hr, 4°C.
- [565] 10 mL 100 mM Tris-HCl pH 7.5 is added to pellet and incubate O/N (overnight) at 4°C.
- [566] The pellet is resuspended and the sample was centrifuged at 120,000xg for 1 hour at 4°C.
- [567] The supernatant is discarded and the pellet was resuspended in a minimal volume of PBS.
- [568] Dosing pmEVs is based on particle counts, as assessed by Nanoparticle Tracking Analysis (NTA) using a NanoSight NS300 (Malvern Panalytical) according to manufacturer instructions. Counts for each sample are based on at least three videos of 30 sec duration each, counting 40-140 particles per frame.
- [569] *Gamma irradiation:* For gamma irradiation, pmEVs are prepared in frozen form and gamma irradiated on dry ice at 25kGy radiation dose; whole microbe lyophilized powder is gamma irradiated at ambient temperature at 17.5kGy radiation dose.
- Lyophilization:* Samples are placed in lyophilization equipment and frozen at -45 °C. The lyophilization cycle included a hold step at -45 °C for 10 min. The vacuum begins and is set to 100 mTorr and the sample was held at -45 °C for another 10 min. Primary drying begins with a temperature ramp to -25 °C over 300 minutes and it is held at this temperature for

4630 min. Secondary drying starts with a temperature ramp to 20 °C over 200 min while the vacuum is decreased to 20 mTorr. It is held at this temperature and pressure for 1200 min. The final step increases the temperature from 20 to 25 °C where it remains at a vacuum of 20 mTorr for 10 min.

Example 38: Oral Delivery of *Harryflintia acetispora* smEVs and Whole Bacterium Strain A in CT26 Tumor Studies

[570] Female 8 week old BALB/c mice were acquired from Taconic Biosciences and allowed to acclimate at a vivarium for 1 weeks. On Day 0, mice were anesthetized with isoflurane, and inoculated subcutaneously on the left flank with 1.0×10^5 CT-26 cells (0.1mL) prepared in PBS and Corning (GFR) Phenol Red-Free Matrigel (1:1). Mice were allowed to rest for 9 days post CT-26 inoculation to allow formation of palpable tumors. On Day 9, tumors were measured using a sliding digital caliper to collect length and width in measurements (in millimeters) to calculate estimated tumor volume ($((L \times W \times W)/2) = TV_{mm^3}$). Mice were randomized into different treatment groups with a total of 9 or 10 mice per group. Randomization was done to balance all treatment groups, allowing each group to begin treatment with a similar average tumor volume and standard deviation. Dosing began on Day 10, and ended on Day 22 for 13 consecutive days of dosing. Mice were orally dosed BID with *H. acetispora* smEVs or parental microbe, or Q4D intraperitoneally with 200ug anti-mouse PD-1 antibody. Body weight and tumor measurements were collected on a MWF (Monday-Wednesday-Friday) schedule. Dose of smEVs was determined by particle count by NTA. Dose of parental microbe was determined by total cell count (TCC).

[571] The Day 22 Tumor Volume Summary in Fig. 13 compares several doses of *H. acetispora* smEV (2×10^5 to 2×10^{11}) against (2) doses of respective parental microbe at matched particle to TCC. Doses of *H. acetispora* smEV (2×10^5 to 2×10^{11}) showed significant efficacy and were not significantly different than anti-PD-1. *H. acetispora* smEV (2×10^3) did not show efficacy. Parental Microbe *H. acetispora* showed significant efficacy at the 2×10^9 dose, but not at the 2×10^7 dose. Treatment with *H. acetispora* smEVs at the 2×10^7 dose was efficacious, and had statistically significant efficacy over the parental microbe *H. acetispora* at the matched dose.

Example 39: Oral Delivery of *Harryflintia acetispora* Strain A smEVs in a FITC**Mouse Model of Cutaneous Hypersensitivity**

[572] Exposure of skin to an antigen can, over time, lead to an allergic response to that antigen, for example, in the form of a skin allergy (e.g. dermatitis or atopic dermatitis or eczema) and/or in the form of a food allergy (see e.g. Han et al. The atopic march: current insights into skin barrier dysfunction and epithelial cell-derived cytokines. *Immunol. Rev.* 2017. July;278(1):116-130. Doi: 10.1111/immr.12546; Kawasaki et al. Skin inflammation exacerbates food allergy symptoms in epicutaneously sensitized mice. *Allergy*. 2018. June;73(6):1313-1321. Doi: 10.1111/all.13404; Martel et al. Translational animal models of atopic dermatitis for preclinical studies. *Yale J. Biol. Med.* 2017. Sept;90(3):389-402; Jin et al. Animal models of atopic dermatitis. *J. Invest. Dermatol.* 2009. Jan: 129(1):31-40. Doi: 10.1038/jid.2008.106).

[573] The effects of *Harryflintia acetispora* Strain A smEVs were studied in a fluorescein isothiocyanate (FITC)-driven contact hypersensitivity model (See e.g. Li et al. T-helper type-2 contact hypersensitivity of Balb/c mice aggravated by dibutyl phthalate via long-term dermal exposure. Feb. 3, 2014 <https://doi.org/10.1371/journal.pone.0087887>; Imai et al. Effects of phthalate esters on the sensitization phase of contact hypersensitivity induced by fluorescein isothiocyanate. *Clin. Exp. Allergy*. 2006 Nov; 36(11): 1462-8; Dearman et al. Role of CD4+ T helper 2-type cells in cutaneous inflammatory responses induced by fluorescein isothiocyanate. *Immunology*. 2000 Dec; 101(4):442-451. Doi: 10.1046/j.1365-2567.2000.01126.x).

[574] *Harryflintia acetispora* Strain A smEVs were administered by oral gavage, and the effects of *Harryflintia acetispora* Strain A smEVs on inflammation were analyzed. Doses were based on particle count.

FITC-driven hypersensitivity Study Protocol:

[575] Mice were purchased from Taconic and allowed to acclimate to the vivarium for at least 1 week prior to the start of the experiment. Mice were housed at 5 animals (or fewer) per cage, with each cage constituting a different treatment group.

[576] On day 0, mice were anesthetized with isoflurane (one at a time) and their backs were shaved.

[577] On day 1, a solution of 0.5 % FITC (w/v) was dissolved in adjuvant (dibutyl phthalate (DBP)) and acetone (1:1). To prepare the 0.5 % FITC, 250mg FITC was dissolved

in 25ml acetone. Once completely dissolved, 25ml of DBP was added and mixed by vortexing.

[578] On days 1 and 2, mice were sensitized on the back by applying 400 μ l of the 0.5 % FITC solution with a pipette. Anaerobic sucrose served as the negative control. Dexamethasone served as the positive control (Dexamethasone stock solution was prepared by resuspending 25mg of dexamethasone (Sigma) in 1.6 ml of 96% ethanol).

[579] On days 1-6, mice were orally gavaged with vehicle (negative control, group 1) or *Harryflintia acetispora* Strain A smEVs (group 3 and 4), or injected intraperitoneally (i.p.) with Dexamethasone (positive control, group 2) according to following study design:

Group	Sensitization 0.5% FITC in 400 μ L	Challenge 0.5% FITC in 20 μ l	Test substance (treatment days 1-6)
1 (n=5)	Day 1, 2	Day 6	Vehicle (anaerobic sucrose)
2 (n=5)	Day 1, 2	Day 6	Dexamethasone (1mg/kg)
3 (n=5)	Day 1, 2	Day 6	<i>Harryflintia acetispora</i> Strain A smEVs (2E+10)
4 (n=5)	Day 1, 2	Day 6	<i>Harryflintia acetispora</i> Strain A smEVs (2E+07)

[580] In addition to the daily gavage (groups 1, 3 and 4) and i.p. injection (group 2), the mice were FITC-challenged on day 6 as follows: On day 6, each mouse was anesthetized with isoflurane and a baseline left ear measurement was obtained using calipers, and then 20 μ l of 0.5% FITC solution was applied on the left ear (20 μ l 0.5% FITC (w/v) DBP:acetone (1:1) ("ear challenge" or "FITC challenge").

[581] On day 7, a 24-hour post ear challenge measurement was obtained using calipers.

[582] Results are shown in Fig. 14. Oral administration of *Harryflintia acetispora* Strain A smEVs significantly reduced ear swelling at 24h post-FITC ear challenge.

Example 40: Oral Delivery of *Megasphaera* sp. Strain A smEVs and *Harryflintia acetispora* Strain A smEVs in a FITC Mouse Model of Cutaneous Hypersensitivity
FITC-driven hypersensitivity Study Protocol:

[583] Mice were purchased from Taconic and allowed to acclimate to the vivarium for at least 1 week prior to the start of the experiment. Mice were housed at 5 animals (or fewer) per cage, with each cage constituting a different treatment group.

[584] On day 0, mice were anesthetized with isoflurane (one at a time) and their backs were shaved.

[585] On day 1, a solution of 0.5 % FITC (w/v) was dissolved in adjuvant (dibutyl phthalate (DBP)) and acetone (1:1). To prepare the 0.5 % FITC, 250mg FITC was dissolved in 25ml acetone. Once completely dissolved, 25ml of DBP was added and mixed by vortexing.

[586] On days 1 and 2, mice were sensitized on the back by applying 400 µl of the 0.5 % FITC solution with a pipette. Anaerobic sucrose served as the negative control. Dexamethasone served as the positive control (Dexamethasone stock solution was prepared by resuspending 25mg of dexamethasone (Sigma) in 1.6 ml of 96% ethanol).

[587] On days 1-6, mice were orally gavaged with vehicle (negative control, group 1) or *Harryflintia acetispora* Strain A smEVs (group 3, 4 and 5), or *Megasphaera* sp. Strain A smEVs (group 6, 7 and 8) or injected intraperitoneally (i.p.) with Dexamethasone (positive control, group 2) according to following study design:

Group	Sensitization 0.5% FITC in 400 µL	Challenge 0.5% FITC in 20ul	Test substance (treatment days 1-6)
1 (n=5)	Day 1, 2	Day 6	Vehicle (anaerobic sucrose)
2 (n=5)	Day 1, 2	Day 6	Dexamethasone (1mg/kg)
3 (n=5)	Day 1, 2	Day 6	<i>Harryflintia acetispora</i> Strain A smEVs (2E+09)
4 (n=5)	Day 1, 2	Day 6	<i>Harryflintia acetispora</i> Strain A smEVs (2E+07)
5 (n=5)	Day 1, 2	Day 6	<i>Harryflintia acetispora</i> Strain A smEVs (2E+05)
6 (n=5)	Day 1, 2	Day 6	<i>Megasphaera</i> sp. Strain A smEVs (2E+09)
7 (n=5)	Day 1, 2	Day 6	<i>Megasphaera</i> sp. Strain A smEVs (2E+07)

8 (n=5)	Day 1, 2	Day 6	<i>Megasphaera</i> sp. Strain A smEVs (2E+05)
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[588] In addition to the daily gavage (groups 1, 3, 4, 5, 6, 7 and 8) and i.p. injection (group 2), the mice were FITC-challenged on day 6 as follows: On day 6, each mouse was anesthetized with isoflurane and a baseline left ear measurement was obtained using calipers, and then 20 μ l of 0.5% FITC solution was applied on the left ear (20 μ l 0.5% FITC (w/v) DBP:acetone (1:1) ("ear challenge" or "FITC challenge").

[589] On day 7, a 24-hour post ear challenge measurement was obtained using calipers. Mice were euthanized and mesenteric lymph nodes single cell suspensions were restimulated with PMA/Ionomycin for 48h and supernatants were collected for downstream cytokine analyses using MSD (Meso Scale Discovery, Catalog# K15069L-2) according to manufacturer's instructions.

[590] Results are shown in Fig. 15. Oral administration of *Harryflintia acetispora* Strain A smEVs and *Megasphaera* sp. Strain A smEVs significantly reduced ear swelling at 24h post-FITC ear challenge.

[591] Compared to the vehicle group, *Harryflintia acetispora* Strain A smEVs and *Megasphaera* sp. Strain A smEVs treatment reduced cytokine levels for IL-4, IL-5, IL-13, IL-10, and IL-33 in the mesenteric lymph nodes (mLNs) (Figs. 16A-16E).

[592] *Megasphaera* sp. Strain A used in this example is *Megasphaera* sp. Strain A (ATCC Deposit Number PTA-126770).

Example 41: *Harryflintia acetispora* Strain A smEVs require engagement of the TLR2 receptor for efficacy

[593] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. Mice were treated with anti-TLR2 (Toll-like receptor 2) antibodies Clone C9A12 (100 μ L IP; 2mg/mL) or a Rat IgG2a isotype control Clone 2A3 (100 μ L IP; 2mg/mL) on days 0, 3, and 6. Mice were then orally gavaged daily with smEVs (100 μ L; 2E11 particles/mL) or dosed intraperitoneally with dexamethasone (100 μ L; 1 mg/kg) from days 5-8. After dosing on day 8, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with

Fowler calipers and the mice were challenged intradermally with KLH in saline (10 µl) in the left ear and ear thickness measurements were taken at 24 hours.

[594] The 24 hour ear measurement results are shown in Fig. 17. This study demonstrates that neutralization of TLR2 by a monoclonal antibody inhibits the anti-inflammatory effects of *Harryflintia acetispora* Strain A smEVs. This demonstrates that for efficacy, smEVs require engagement of the TLR2 receptor in the small intestine.

Example 42: *Harryflintia acetispora* Strain A smEVs require engagement of the IL-10 receptor for efficacy

[595] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. Mice were treated with anti-IL-10R antibodies Clone 1B1.3A (100 µL IP; 2mg/mL) or a Rat IgG2a isotype control Clone MOPC-21 (100 µL IP; 2mg/mL) on days 0, 3, and 6. Mice were then orally gavaged daily with smEVs (100 µL; 2E11 particles/mL) or dosed intraperitoneally with dexamethasone (100 µL; 1 mg/kg) from days 5-8. After dosing on day 8, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 µl) in the left ear and ear thickness measurements were taken at 24 hours.

[596] The 24 hour ear measurement results are shown in Fig. 18. This study demonstrates that neutralization of the IL-10 receptor (IL-10R) by a monoclonal antibody inhibits the anti-inflammatory effects of *Harryflintia acetispora* Strain A smEVs. This demonstrates that the smEVs induce release of IL-10 which is required for anti-inflammatory effects.

Example 43: Lymphocyte gut homing needed for *Harryflintia acetispora* Strain A smEVs efficacy

[597] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. To block lymphocyte homing to the gut and gut-associated lymphoid tissues, mice were treated with a combination of anti-CD62L and anti-α4β7 antibodies Clones Mel-14 and DATK32 respectively (100 µL IP; 2.5mg/mL each antibody) or a Rat IgG2a isotype control Clone MOPC-21 (100 µL IP; 5mg/mL) on

days 1, 3, 5, and 7. Mice were then orally gavaged daily with smEVs (100 μ L; 2E11 particles/mL) or dosed intraperitoneally with dexamethasone (100 μ L; 1 mg/kg) from days 5-8. On day 13, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 μ l) in the left ear and ear thickness measurements were taken at 24 hours.

[598] The 24 hour ear measurement results are shown in Fig. 19. This study demonstrates that when lymphocytes are unable to home to the lymphoid tissue within the gut, *Harryflintia acetispora* Strain A smEVs cannot induce anti-inflammatory effects. This suggests that the smEVs induce modulation of lymphocytes to a more anti-inflammatory state in the gut lymphoid tissue, leading to anti-inflammatory effects in the periphery.

Example 44: B cells needed for *Harryflintia acetispora* Strain A smEVs efficacy

[599] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated a vivarium for one week. Mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization on day 0. To deplete B cells, mice were treated with anti-CD20 Clone SA271G2 (100 μ L IP; 2mg/mL) or a Rat IgG2b isotype control Clone MPC-11 (100 μ L IP; 2mg/mL) on days 0, 3, 6, 9 and 12. Mice were then orally gavaged daily with smEVs (100 μ L; 2E11 particles/mL) or dosed intraperitoneally with dexamethasone (100 μ L; 1 mg/kg) from days 5-8. On day 13, mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 μ l) in the left ear and ear thickness measurements were taken at 24 hours.

[600] The 24 hour ear measurement results are shown in Fig. 20. This study demonstrates that when B cells are depleted, *Harryflintia acetispora* Strain A smEVs cannot induce anti-inflammatory effects. This suggests that the smEVs induce modulation of B cell lymphocytes that are critical for mediating anti-inflammatory effects in the periphery.

Example 45: Adoptive T cell transfer from mice treated with *Harryflintia acetispora* Strain A smEVs

[601] Female 5 week old C57BL/6 mice were purchased from Taconic Biosciences and acclimated at a vivarium for one week. Mice were separated into two stages, Stage 1 (donor mice) and Stage 2 (recipient mice). Stage 1 mice were primed with an emulsion of

KLH and CFA (1:1) by subcutaneous immunization on day 0. Mice were then orally gavaged daily with smEVs (100 μ L; 2E11 particles/mL) from days 5-8. On day 5, Stage 2 mice were primed with an emulsion of KLH and CFA (1:1) by subcutaneous immunization. On day 9, Stage 1 mice were sacrificed and spleens/lymph nodes were harvested. Tissues were pooled, homogenized, and then CD4+ T cells were isolated by negative selection and diluted to 83.3×10^6 cells/mL in PBS. Isolated CD4+ T cells were then transferred into Stage 2 mice (100 μ L; IP). On day 12, Stage 2 mice were anaesthetized with isoflurane, left ears were measured for baseline measurements with Fowler calipers and the mice were challenged intradermally with KLH in saline (10 μ l) in the left ear and ear thickness measurements were taken at 24 hours.

[602] The 24 hour ear measurement results are shown in Fig. 21. Mice that received CD4+ T cells from mice dosed with *Harryflintia acetispora* Strain A smEVs were able to confer anti-inflammatory effects in the recipient mice that had not received the smEVs. This study demonstrates that dosing mice with *Harryflintia acetispora* Strain A smEVs induces modulation of CD4+ T cells to an anti-inflammatory state that can be transferred into mice that have not been dosed with the smEVs.

Incorporation by Reference

[603] All publications or patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

[604] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A pharmaceutical composition comprising *Harryflintia acetispora* bacteria.
2. The pharmaceutical composition of claim 1, wherein the *Harryflintia acetispora* is a strain comprising at least 90% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
3. The pharmaceutical composition of claim 1, wherein the *Harryflintia acetispora* is a strain comprising at least 99% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
4. The pharmaceutical composition of claim 1, wherein the *Harryflintia acetispora* is a strain comprising at least 99% 16S sequence identity to SEQ ID NO: 1.
5. The pharmaceutical composition of claim 1, wherein the *Harryflintia acetispora* is *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
6. The pharmaceutical composition of any one of claims 1-5, wherein at least 50% of the bacteria in the pharmaceutical composition are *Harryflintia acetispora* Strain A.
7. The pharmaceutical composition of any one of claims 1-6, wherein at least 90% of the bacteria in the pharmaceutical composition are *Harryflintia acetispora* Strain A.
8. The pharmaceutical composition of any one of claims 1-7, wherein substantially all of the bacteria in the pharmaceutical composition are *Harryflintia acetispora* Strain A.
9. The pharmaceutical composition of any one of claims 1-8, wherein the bacterial composition comprises at least 1×10^6 total cells of *Harryflintia acetispora* Strain A.
10. The pharmaceutical composition of any one of claims 1-9, wherein the bacterial composition comprises at least 1×10^7 total cells of *Harryflintia acetispora* Strain A.
11. The pharmaceutical composition of any one of claims 1-10, wherein the bacterial composition comprises at least 1×10^8 total cells of *Harryflintia acetispora* Strain A.
12. The pharmaceutical composition of any one of claims 1-11, wherein the pharmaceutical composition comprises live bacteria.
13. The pharmaceutical composition of any one of claims 1-11, wherein the pharmaceutical composition comprises attenuated bacteria.
14. The pharmaceutical composition of any one of claims 1-11, wherein the pharmaceutical composition comprises killed bacteria.

15. The pharmaceutical composition of any one of claims 1-14, wherein the pharmaceutical composition comprises lyophilized bacteria.
16. The pharmaceutical composition of any one of claims 1-15, wherein the pharmaceutical composition comprises irradiated bacteria.
17. The pharmaceutical composition of claim 16, wherein the pharmaceutical composition comprises gamma irradiated bacteria.
18. A pharmaceutical composition comprising isolated extracellular vesicles (mEVs) produced from *Harryflintia acetispora*.
19. The pharmaceutical composition of claim 18, wherein the *Harryflintia acetispora* is a strain comprising at least 90% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
20. The pharmaceutical composition of claim 18, wherein the *Harryflintia acetispora* is a strain comprising at least 99% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
21. The pharmaceutical composition of claim 18, wherein the *Harryflintia acetispora* is a strain comprising at least 99% 16S sequence identity to SEQ ID NO: 1.
22. The pharmaceutical composition of claim 18, wherein the *Harryflintia acetispora* is *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
23. The pharmaceutical composition of claim 18, wherein at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% of the pharmaceutical composition is mEVs.
24. The pharmaceutical composition of any one of claims 18-23, wherein the composition comprises secreted mEVs (smEVs).
25. The pharmaceutical composition of any one of claims 18-23, wherein the composition comprises processed mEVs (pmEVs).
26. The pharmaceutical composition of any one of claims 18-23, wherein the mEVs comprise pmEVs and the pmEVs are produced from bacteria that have been gamma irradiated, UV irradiated, heat inactivated, acid treated or oxygen sparged.
27. The pharmaceutical composition of any one of claims 18-23, wherein the mEVs comprise pmEVs and the pmEVs are produced from live bacteria.

28. The pharmaceutical composition of any one of claims 18-27, wherein the mEVs are lyophilized (e.g., the lyophilized product further comprises a pharmaceutically acceptable excipient).
29. The pharmaceutical composition of any one of claims 18-28, wherein the mEVs are gamma irradiated.
30. The pharmaceutical composition of any one of claims 18-28, wherein the mEVs are UV irradiated.
31. The pharmaceutical composition of any one of claims 18-28, wherein the mEVs are heat inactivated (e.g., at 50°C for two hours or at 90°C for two hours).
32. The pharmaceutical composition of any one of claims 18-28, wherein the mEVs are acid treated.
33. The pharmaceutical composition of any one of claims 18-28, wherein the mEVs are oxygen sparged (e.g., at 0.1 vvm for two hours).
34. The pharmaceutical composition of any one of claims 18-33, wherein the dose of mEVs is about 2×10^6 to about 2×10^{16} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).
35. The pharmaceutical composition of any one of claims 18-34, wherein the dose of mEVs is about 5 mg to about 900 mg total protein (e.g., wherein total protein is determined by Bradford assay or BCA assay).
36. A pharmaceutical composition comprising *Harryflintia acetispora* microbial extracellular vesicles (mEVs) and *Harryflintia acetispora* bacteria.
37. The pharmaceutical composition of claim 36, wherein the *Harryflintia acetispora* is a strain comprising at least 90% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
38. The pharmaceutical composition of claim 36, wherein the *Harryflintia acetispora* is a strain comprising at least 99% genomic, 16S, and/or CRISPR sequence identity to the nucleotide sequence of the *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).
39. The pharmaceutical composition of claim 36, wherein the *Harryflintia acetispora* is a strain comprising at least 99% 16S sequence identity to SEQ ID NO: 1.
40. The pharmaceutical composition of claim 36, wherein the *Harryflintia acetispora* is *Harryflintia acetispora* Strain A (ATCC Deposit Number PTA-126694).

41. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are

Harryflintia acetispora mEVs.

42. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total particles in the pharmaceutical composition are

Harryflintia acetispora bacteria particles.

43. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total proteins in the pharmaceutical composition are

Harryflintia acetispora mEVs.

44. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%,

80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total proteins in the pharmaceutical composition are *Harryflintia acetispora* bacteria proteins.

45. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* mEVs.

46. The pharmaceutical composition of any one of claims 36-40, wherein at least, about, or no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the total lipids in the pharmaceutical composition are *Harryflintia acetispora* bacteria lipids.

47. The pharmaceutical composition of any one of claims 1-46, wherein the pharmaceutical composition is for the treatment of a disease.

48. The pharmaceutical composition of any one of claims 1-47, wherein the pharmaceutical composition is for the treatment of an immune disorder (e.g., a cancer, an autoimmune disease, an inflammatory disease, or a metabolic disease).

49. The pharmaceutical composition of any one of claims 1-47, wherein the pharmaceutical composition is for the treatment of an inflammatory disorder (e.g., dermatitis).

50. The pharmaceutical composition of any one of claims 1-47, wherein the pharmaceutical composition is for the treatment of a cancer (e.g., colorectal cancer).

51. The pharmaceutical composition of any one of claims 1-47, wherein the pharmaceutical composition is for the treatment of a dysbiosis.

52. The pharmaceutical composition of any one of claims 1-51, wherein the pharmaceutical composition induces an immune response.
53. The pharmaceutical composition of any one of claims 1-52, wherein the pharmaceutical composition activates innate antigen presenting cells.
54. The pharmaceutical composition of any one of claims 1-53, wherein the pharmaceutical composition is formulated for oral, rectal, sublingual, intradermal, intraperitoneal, or subcutaneous administration.
55. The pharmaceutical composition of any one of claims 1-54, wherein the pharmaceutical composition has one or more beneficial immune effects outside the gastrointestinal tract, e.g., when orally administered.
56. The pharmaceutical composition of any one of claims 1-55, wherein the pharmaceutical composition modulates immune effects outside the gastrointestinal tract in the subject, e.g., when orally administered.
57. The pharmaceutical composition of any one of claims 1-56, wherein the pharmaceutical composition comprises a solid dose form.
58. The pharmaceutical composition of claim 57, wherein the solid dose form comprises a tablet, a minitab, a capsule, a pill, or a powder, or a combination of the foregoing.
59. The pharmaceutical composition of claim 57 or 58, wherein the solid dose form further comprises a pharmaceutically acceptable excipient.
60. The pharmaceutical composition of any one of claims 57-59, wherein the solid dose form comprises an enteric coating.
61. The pharmaceutical composition of any one of claims 57-60, wherein the solid dose form is for oral administration.
62. The pharmaceutical composition of any one of claims 1-56, wherein the pharmaceutical composition comprises a suspension.
63. The pharmaceutical composition of claim 62, wherein the suspension is for oral administration (e.g., the suspension comprises PBS, and optionally, sucrose or glucose).
64. The pharmaceutical composition of claim 62, wherein the suspension is for intravenous administration (e.g., the suspension comprises PBS).
65. The pharmaceutical composition of claim 62, wherein the suspension is for intraperitoneal administration (e.g., the suspension comprises PBS).
66. The pharmaceutical composition of claim 62, wherein the suspension is for intratumoral administration (e.g., the suspension comprises PBS).

67. The pharmaceutical composition of any one of claims 62-66, wherein the suspension further comprises a pharmaceutically acceptable excipient.
68. The pharmaceutical composition of any one of claims 62-67, wherein the suspension further comprises a buffer (e.g., PBS).
69. The pharmaceutical composition of any one of claims 1-68, wherein the composition further comprises one or more additional therapeutic agents.
70. The pharmaceutical composition of any one of claims 1-69, wherein the pharmaceutical composition is formulated for a daily dose.
71. The pharmaceutical composition of any one of claims 1-69, wherein the pharmaceutical composition is formulated for twice a day dose, wherein each dose is half of the daily dose.
72. The pharmaceutical composition of any one of claims 1-71 for use in treating a disease (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease).
73. Use of a pharmaceutical composition of any one of claims 1-71 for the preparation of a medicament for the treatment of a disease (e.g., a cancer, an autoimmune disease, an inflammatory disease, a dysbiosis, or a metabolic disease).
74. A method of treating a subject (e.g., human) in need thereof, comprising administering to the subject a pharmaceutical composition of any one of claims 1-71.
75. The method of claim 74, wherein the subject is in need of treatment for an immune disorder or a metabolic disease.
76. The method of claim 74, wherein the subject is in need of treatment for a cancer.
77. The method of claim 74, wherein the subject is in need of treatment for an inflammatory disease.
78. The method of claim 74, wherein the subject is in need of treatment for a dysbiosis.
79. The method of any one of claims 74-78, further comprising administering to the subject an additional therapeutic agent.
80. The method of any one of claims 74-79, wherein the pharmaceutical composition is administered intravenously.
81. The method of any one of claims 74-79, wherein the pharmaceutical composition is administered intratumorally.
82. The method of any one of claims 74-79, wherein the pharmaceutical composition is administered subcutaneously.

83. The method of any one of claims 74-79, wherein the pharmaceutical composition is administered by injection, e.g., subcutaneous, intradermal, or intraperitoneal injection.
84. The method of any one of claims 74-79, wherein the pharmaceutical composition is administered orally.
85. The method of any one of claims 74-84, wherein the pharmaceutical composition further comprises one or more additional therapeutic agents.
86. The method of any one of claims 74-85, wherein the dose of mEVs in the pharmaceutical composition is about 2×10^6 to about 2×10^{16} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).
87. The method of any one of claims 74-86, wherein the dose of mEVs in the pharmaceutical composition is 5 mg to about 900 mg total protein (e.g., wherein total protein is determined by Bradford assay or BCA assay).
88. The method of any one of claims 74-87, wherein the pharmaceutical composition is administered once a day.
89. The method of any one of claims 74-87, wherein the pharmaceutical composition is administered twice a day.
90. The method of any one of claims 74-87, wherein the pharmaceutical composition is formulated for a daily dose.
91. The method of any one of claims 74-87, wherein the pharmaceutical composition is formulated for twice a day dose, wherein each dose is half of the daily dose.
92. A method for preparing a pharmaceutical composition of any one of claims 1-71 in a suspension, the method comprising: combining mEVs, bacteria, or any combination thereof, with a pharmaceutically acceptable buffer (e.g., PBS); thereby preparing the pharmaceutical composition.
93. The method of claim 92, wherein the suspension further comprises sucrose or glucose.
94. The method of claim 92 or 93, wherein the suspension is for oral administration.
95. The method of claim 92 or 93, wherein the suspension is for intravenous administration.
96. The method of claim 92 or 93, wherein the suspension is for intraperitoneal administration.
97. The method of claim 92 or 93, wherein the suspension is for intratumoral administration.

98. The method of any one of claims 92-97, wherein the suspension further comprises a pharmaceutically acceptable excipient.
99. The method of any one of claims 92-98, wherein the suspension further comprises a buffer (e.g., PBS).
100. The method of any one of claims 92-99, wherein the composition further comprises one or more additional therapeutic agents.
101. The method of any one of claims 92-100, wherein the pharmaceutical composition is administered orally.
102. The method of any one of claims 92-100, wherein the pharmaceutical composition is administered intravenously.
103. The method of any one of claims 92-100, wherein the pharmaceutical composition is administered intratumorally.
104. The method of any one of claims 92-100, wherein the pharmaceutical composition is administered subcutaneously.
105. The method of any one of claims 92-100, wherein the pharmaceutical composition is administered by injection, e.g., subcutaneous, intradermal, or intraperitoneal injection.
106. The method of any one of claims 92-105, wherein the dose of mEVs in the pharmaceutical composition is about 2×10^6 to about 2×10^{16} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).
107. The method of any one of claims 92-106, wherein the dose of mEVs in the pharmaceutical composition is 5 mg to about 900 mg total protein (e.g., wherein total protein is determined by Bradford assay or BCA assay).
108. A pharmaceutical composition prepared by the method of any one of claims 92-107.
109. A method for preparing a pharmaceutical composition of any one of claims 1-71 in a solid dose form, the method comprising:
- a) combining the mEVs, bacteria, or any combination thereof, of any one of claims 1-71 with a pharmaceutically acceptable excipient, and
 - b) compressing the mEVs, bacteria, or any combination thereof, and a pharmaceutically acceptable excipient, thereby preparing the pharmaceutical composition.
110. The method of claim 109, wherein the method further comprises enterically coating the solid dose form.
111. The method of claim 109 or 110, wherein the solid dose form comprises a tablet, a minitab, a capsule, a pill, or a powder, or a combination of the foregoing.

112. The method of any one of claims 109-111, wherein the composition further comprises one or more additional therapeutic agents.
113. The method of any one of claims 109-112, wherein the pharmaceutical composition is administered orally.
114. The method of any one of claims 109-113, wherein the dose of mEVs in the pharmaceutical composition is about 2×10^6 to about 2×10^{16} particles (e.g., wherein particle count is determined by NTA (nanoparticle tracking analysis)).
115. The method of any one of claims 109-114, wherein the dose of mEVs in the pharmaceutical composition is 5 mg to about 900 mg total protein (e.g., wherein total protein is determined by Bradford assay or BCA assay).
116. A pharmaceutical composition prepared by the method of any one of claims 109-115.