COMPOSITIONS AND METHODS FOR LYOPHILIZATION OF BACTERIA OR *LISTERIA* STRAINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Application No. 62/560,318, filed September 19, 2017, herein incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS WEB

[0002] The Sequence Listing written in file 519152SEQLIST.txt is 89.2 kilobytes, was created on August 24, 2018, and is hereby incorporated by reference.

BACKGROUND

[0003] Lyophilization is a process that removes solvent from a solution to form solid or powder that is stable and easier to store at elevated temperature than liquid. Lyophilization, also known as freeze drying, involves freezing followed by sublimation. The resulting lyophilized matter may be stored without refrigeration or at higher temperatures than liquid, reducing storage and transportation costs of the substance as well as the storage space required for the product. It also can reduce the weight of the product, which similarly reduces shipping and related costs. Lyophilization is particularly useful for preserving and storing various biological molecules, because it increases their shelf-life.

[0004] Compared to liquid formulations, solid formulations have multiple advantages such as superior storage stability, reduced molecular mobility and unwanted chemical reactions, and less package weight for increased ease in shipping and distribution. Furthermore, as all currently available commercial vaccines require low temperature storage, the goal is to utilize solid state stabilization techniques to enhance their room temperature or high temperature stability and to reduce the reliance on cold-chain to maintain efficacy and ensure safety. Although lyophilization is the preferred preservation method because the low storage and transport costs of freeze-dried bacterial cultures are a major advantage compared to cryopreservation, lyophilization is a very complex physical process affected by many parameters requiring specific equipment and trained personnel. Freeze-drying can cause many types of damage to cells,

including a loss of viability, reduction of metabolic activity, and changes in cell morphology, which can affect the physiology, characterization, and functions of cells such as bacteria.

[0005] In addition, the effect of varying different lyophilization parameters is highly strain-specific, and this strain dependency makes it difficult to draw general conclusions or guidelines from any one particular strain. The high biological and metabolic diversity of bacteria makes it difficult and laborious to develop strain-specific optimized freeze-drying procedures. There are very limited data on the lyophilization of *Listeria* bacteria strains such as *Listeria monocytogenes* and what parameters need to be optimized and how to optimize them to make lyophilization a viable option for *Listeria*.

SUMMARY

[0006] Methods and compositions are provided for lyophilization of bacteria or *Listeria* strains, such as *Listeria monocytogenes*. In one aspect, provided are methods for producing a lyophilized composition comprising a bacteria or *Listeria* strain. Some such methods can comprise providing a composition comprising a bacteria or *Listeria* strain in a formulation comprising a buffer, cooling the composition in a freezing step, exposing the cooled composition to a vacuum and a first increased temperature in a primary drying step, and exposing the composition from the primary drying step to a vacuum and a second increased temperature in a secondary drying step, whereby the lyophilized composition is produced.

[0007] In some such methods, the bacteria or *Listeria* strain used in the composition is a frozen *Listeria* strain that is thawed prior to the freezing step. In a specific example, the frozen bacteria or *Listeria* strain can be thawed at a temperature of about 2°C to about 37°C, about 20°C to about 37°C, about 23°C to about 37°C, about 25°C to about 37°C, about 32°C to about 37°C, or about 37°C. Optionally, the thawing is for no more than about 8 hours. Optionally, the thawed bacteria or *Listeria* strain is held at temperature of between about 2°C and about 8°C for no more than about 24 hours. In a specific example, the concentration of the bacteria or *Listeria* strain being thawed can be between about 1x10E9 and about 1x10E10 colony forming units (CFU) per milliliter.

[0008] In some such methods, the formulation comprises a buffer and sucrose. For example, the formulation buffer can comprise about 1% to about 5% w/v sucrose, about 2% to about 3% w/v sucrose, or about 2.5% w/v sucrose. Optionally, the formulation does not comprise one or

more other excipients such as trehalose, monosodium glutamate (MSG), or recombinant human serum albumin (rHSA).

[0009] In some such methods, the formulation comprises about 1x10E9 to about 1x10E10 colony forming units (CFU) of bacteria or *Listeria* per milliliter.

[0010] In some such methods, the holding temperature in the primary drying step is between about −10°C and about −30°C, between about −12°C and about −22°C, between about −17°C and about −19°C, or about −18°C.

[0011] In some such methods, the residual moisture in the lyophilized composition is at least about 2.5%, at least about 3%, or at least about 3.5%. In some such methods, the residual moisture is between about 1% and about 5% or between about 2% and about 4%.

[0012] In some such methods, the lyophilized composition shows at least about 60%, 70%, 80%, or 90% viability after storage at between about −20°C and about 4°C or after storage at about −20°C or about 4°C for about 6 months, 12 months, 18 months, or 24 months.

[0013] Such methods can comprise, for example: (a) providing a composition comprising a *Listeria* strain in a formulation comprising a buffer and sucrose; (b) cooling the composition provided in step (a) to a holding temperature between about −32°C and about −80°C in a freezing step; (c) exposing the composition produced by step (b) to a vacuum at a holding temperature between about −10°C and about −30°C in a primary drying step; and (d) exposing the composition produced by step (c) to a vacuum at a holding temperature between about −5°C and about 25°C in a secondary drying step whereby the lyophilized composition is produced. Such methods can alternatively comprise, for example: (a) providing a composition comprising a *Listeria* strain in a formulation comprising a buffer and sucrose; (b) cooling the composition provided in step (a) to a holding temperature between about −32°C and about −80°C in a freezing step; (c) exposing the composition produced by step (b) to a vacuum at a holding temperature between about −10°C and about −30°C in a primary drying step; and (d) exposing the composition produced by step (c) to a vacuum at a holding temperature between about 5°C and about 25°C in a secondary drying step whereby the lyophilized composition is produced. In some such methods, the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, a stress response is induced in the *Listeria* strain by exposing the *Listeria* strain to a decreased temperature, the buffer is a phosphate buffer, the formulation comprises 2% to 3% w/v sucrose, the formulation does not comprise trehalose, MSG, or rHSA, the temperature in the primary

drying step (c) is between −17°C and −19°C, and the residual moisture in the lyophilized composition is between 3% and 4%. Some such methods have one or more or all of the following elements: the *Listeria* strain is a recombinant *Listeria monocytogenes* strain; the buffer is a phosphate buffer; the formulation comprises about 2% to about 3% w/v sucrose; the formulation does not comprise trehalose, MSG, or rHSA; the formulation comprises about 1x10E9 to about 1x10E10 colony forming units (CFU) of *Listeria* per milliliter; the holding temperature in the freezing step (a) is between about −40°C and about −50°C; the holding temperature in the primary drying step (c) is between about −17°C and about −19°C; the holding temperature in the secondary drying step (d) is between −1°C and 1°C; and the residual moisture in the lyophilized composition is between about 2.5% and about 4%. In some such methods, the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that is thawed prior to step (a). Optionally, such methods have one or more or all of the following elements: the concentration of the frozen *Listeria* strain being thawed is between about 1x10E9 to about 1x10E10 colony forming units (CFU) per milliliter; the frozen *Listeria* strain is thawed at about 37°C; the frozen *Listeria* strain is thawed for no more than 8 hours; and the frozen *Listeria* strain is held at about 2°C to about 8°C for no more than 24 hours after thawing. Also provided are lyophilized bacteria or *Listeria* strains produced by the lyophilization methods disclosed herein.

[0014] In another aspect, provided are formulations for lyophilization comprising a bacteria or *Listeria* strain. Such formulations can comprise, for example: (1) the *Listeria* strain; (2) a phosphate buffer; and (3) sucrose. In some such formulations, the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, the formulation comprises about 2% to about 3% w/v sucrose, and the formulation does not comprise trehalose, MSG, or rHSA.

[0015] In another aspect, provided are lyophilized compositions comprising a bacteria or *Listeria* strain. Some such lyophilized compositions have a residual moisture of at least about 2.5% or at least about 3%. Some such lyophilized compositions can further comprise a phosphate buffer and sucrose. In some such lyophilized compositions, the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, the lyophilized composition does not comprise trehalose, MSG, or rHSA, and the residual moisture in the lyophilized composition is between 3% and 4%.

[0016] In another aspect, provided are methods of preparing a frozen *Listeria* strain for lyophilization, comprising thawing the frozen *Listeria* strain at a temperature between about

20°C and about 37°C. Optionally, such methods have one or more or all of the following elements: the concentration of the frozen *Listeria* strain being thawed is between about 1x10E9 to about 1x10E10 colony forming units (CFU) per milliliter; the frozen *Listeria* strain is thawed at about 37°C; the frozen *Listeria* strain is thawed for no more than 8 hours; and the frozen *Listeria* strain is held at about 2°C to about 8°C for no more than 24 hours after thawing.

BRIEF DESCRIPTION OF THE FIGURES

[0017] **FIG. 1** shows viable cell count (VCC) data for different formulation buffers (citrate, phosphate, and MOPS (3-(*N*-morpholino)propanesulfonic acid)) and for different ratios of sucrose (Suc) to trehalose (Treh) to monosodium glutamate (MSG) to recombinant human serum albumin (rHSA) over time.

[0018] **FIG. 2A** and **2B** show VCC multivariate data analysis (MVDA) in an accelerated stability study at a 1 month stability anchor point (**FIG. 2A**) and at a 6 month stability anchor point (**FIG. 2B**).

[0019] **FIG. 3** shows VCC data for different OD levels and stabilizer combinations. Heading for each plot indicates OD in vial (2.0, 3.0, 10.0, 12.5, 15.0, 17.5, or 20.0), stabilizer (buffer 2 (2.5% sucrose) or buffer 5 (5% sucrose with amino acid mix).

[0020] **FIG. 4** shows VCC data for different OD levels and ratios of sucrose (Suc) to amino acid mix (AA Mix) to recombinant human serum albumin (rHSA). Heading for each plot indicates OD in vial (2 or 10), sucrose (10, 5, 2.5):amino acid mix (presence (1) or absence(0)):rHSA (0, 1, or 2.5).

[0021] **FIG. 5** shows residual moisture (RM) data for different OD levels and ratios of sucrose (Suc) to amino acid mix (AA Mix) to recombinant human serum albumin (rHSA).

[0022] **FIG. 6** shows residual moisture data for different percentages of sucrose (weight per volume) at different time points in the lyophilization cycle.

[0023] **FIG. 7A** and **7B** show VCC data post-lyophilization after storage at different temperatures for different amounts of time in *Lm* samples after the primary drying step, after the ramp, or after the secondary drying step in the Lyo4 experiment. **FIG. 7A** shows results for formulations having 2.5% weight per volume (w/v) sucrose. **FIG. 7B** shows results for formulations having 5% w/v sucrose.

[0024] **FIG. 8** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in *Lm* samples stressed with a temperature shift in an ice bath prior to lyophilization, *Lm* samples stressed with acid treatment to lower pH prior to lyophilization, *Lm* samples stressed with both a temperature shift and a pH shift prior to lyophilization, and *Lm* samples with no temperature shift or pH shift in the Lyo5 experiment.

[0025] **FIG. 9** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization (Initial) and post-lyophilization (all other samples) after storage at different temperatures for different amounts of time in the Lyo6 experiment.

[0026] **FIG. 10** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization and post-lyophilization after storage at different temperatures for different amounts of time in the Lyo7 experiment.

[0027] **FIG. 11** shows VCC data (percent of average pre-lyophilization VCC) post-lyophilization after storage at different temperatures for different amounts of time for fresh *Lm* samples (Part A) and frozen *Lm* samples that were thawed (Part B) in the Lyo8 experiment.

[0028] **FIG. 12** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in the Lyo9 experiment.

[0029] **FIG. 13** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in *Lm* samples stressed with a temperature shift in an ice bath prior to lyophilization (Part B) and *Lm* samples with no temperature shift (Part A) in the Lyo10 experiment.

[0030] **FIG. 14** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in the Lyo11 experiment.

[0031] **FIG. 15** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in fresh *Lm* samples (Part A), frozen *Lm* samples thawed at 2-8°C prior to lyophilization (Part B), and frozen *Lm* samples thawed at 37°C and incubated 4 hours prior to lyophilization (Part C) in the Lyo12 experiment.

[0032] **FIG. 16** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in fresh *Lm* samples (Part A) and *Lm* samples stored at 2-8°C for 3 days.

[0033] **FIG. 17** shows VCC data post-lyophilization after storage at different temperatures for different amounts of time in *Lm* samples under the following conditions: 2R vials, 1×10^9 VCC, and 1.2mL fill.

[0034] **FIG. 18** shows VCC data (CFU/mL) before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days at 30°C (T_{liq}, T_{lyo}, T42h, T48h, and T72h, respectively).

[0035] **FIG. 19** shows residual moisture content (RM) as a function of the shelf temperature in the secondary drying step (SD temperature).

[0036] **FIG. 20** shows the drug substance manufacturing process flow for Axalimogene Filolisbac (ADXS-HPV).

[0037] **FIG. 21** shows a flow diagram for manufacture of the Axalimogene Filolisbac (ADXS-HPV) drug product.

[0038] **FIG. 22A** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization and post-lyophilization after storage at different temperatures for different amounts of time in the Lyo1 experiment.

[0039] **FIG. 22B** shows residual moisture immediately after lyophilization and after 6 months at 2-8°C in the Lyo1 experiment.

[0040] **FIG. 23A** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization and post-lyophilization after storage at different temperatures for different amounts of time (months) in the Lyo2 experiment.

[0041] **FIG. 23B** shows residual moisture immediately after lyophilization and after 6 months at 2-8°C in the Lyo2 experiment.

[0042] **FIG. 24A** shows residual moisture (RM) using 2.5% sucrose after primary drying, after ramp, and after secondary drying after storage at different temperatures for different amounts of time in the Lyo4 experiment.

[0043] **FIG. 24B** shows residual moisture (RM) using 5.0% sucrose after primary drying, after ramp, and after secondary drying after storage at different temperatures for different amounts of time in the Lyo4 experiment.

[0044] **FIG. 25** shows residual moisture after various stress treatments after storage at different temperatures for different amounts of time in the Lyo5 experiment.

[0045] **FIG. 26** shows residual moisture following temperature shift treatment pre-lyophilization after storage at different temperatures for different amounts of time in the Lyo6 experiment.

[0046] **FIG. 27** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo7 experiment.

[0047] **FIG. 28** shows residual moisture for samples were lyophilized immediately (part A) or samples that were frozen, thawed, and then lyophilized (part B) after storage at different temperatures for different amounts of time in the Lyo8 experiment.

[0048] **FIG. 29** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo9 experiment.

[0049] **FIG. 30** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo10 experiment.

[0050] **FIG. 31** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo11 experiment.

[0051] **FIG. 32** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo12 experiment.

[0052] **FIG. 33** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo13 experiment.

[0053] **FIG. 34** shows residual moisture after storage at different temperatures for different amounts of time in the Lyo14 experiment.

[0054] **FIG. 35** shows VCC data post-lyophilization as a percent of pre-lyophilization after storage at 30°C for different amounts of time in the batch scale experiment.

[0055] **FIG. 36** shows VCC data post-lyophilization as a percent of post-lyophilization after storage at 30°C for different amounts of time in the batch scale experiment.

[0056] **FIG. 37** shows residual moisture (RM) vs. sample in the batch scale experiment.

[0057] **FIG. 38** shows residual moisture (RM) after lyophilization and storage for 72 hours at 30°C in the batch scale experiment.

[0058] **FIG. 39** shows bioactivity and INFγ induction of the lyophilized product compared to non-lyophilized bacteria and 10-mer control in the Lyo11 experiment.

[0059] **FIGS. 40A-B** show VCC data (A) before lyophilization and post-lyophilization immediately after lyophilization and after storage at 30°C for 24, 48, or 72 hours, and residual moisture (B) in the WP3 experiment.

[0060] **FIGS. 41A-B** shows VCC data (A) before lyophilization and post-lyophilization immediately after lyophilization and after storage at 30°C for 24, 48, or 72 hours, and residual moisture (B) in the WP6 experiment.

[0061] **FIGS. 42A-B** show lyophilization cakes (A) and reconstitution times (B) post-lyophilization immediately after lyophilization and after storage at 30°C for 24, 48, or 72 hours in the WP7 experiment.

[0062] **FIG. 43** shows VCC data before lyophilization and post-lyophilization immediately after lyophilization and after storage at 30°C for 24 hours, 72 hours, and 7 days in the WP7 experiment.

[0063] **FIGS. 44A-B** show VCC data (A) and percent live cells (B) vs. storage time at 30°C in the WP7 experiment.

[0064] **FIGS. 45A-B** show CFU/mL (A) and percent viable cells (B) vs. time in the WP7 experiment.

[0065] **FIGS. 46A-B** show VCC data before lyophilization and post-lyophilization immediately after lyophilization and after storage at 30°C for 24 and 72 hours in the WP7 experiment.

[0066] **FIGS. 47A-B** show VCC data before lyophilization and post-lyophilization immediately after lyophilization and after storage at 30°C for 24 and 72 hours in the WP7 experiment.

[0067] **FIG. 48** shows a scatterplot of VCC and RM for ADXS11-001 Pilot Batch.

[0068] **FIG. 49** shows a scatterplot of VCC for Lot# 5329PD-17-01 (ADXS11-001 Pilot Batch).

[0069] **FIG. 50** show a scatterplot of % live for Lot# 5329PD-17-01 (ADXS11-001 Pilot Batch).

[0070] **FIG. 51** show a scatterplot of pH for Lot# 5329PD-17-01 (ADXS11-001 Pilot Batch).

[0071] **FIG. 52** shows a scatterplot of VCC for Lot# 5329PD-17-01 stored at 30°C (ADXS11-001 Pilot Batch).

[0072] **FIG. 53** shows a scatterplot of % live for Lot# 5329PD-17-01 stored at 30°C (ADXS11-001 Pilot Batch).

[0073] **FIG. 54** shows a chart illustrating implantation and dosing schedule (ADXS11-001 Pilot Batch).

[0074] **FIG. 55** shows graphs illustrating both lyophilized AXAL and clinical AXAL inhibit tumor growth in the TC-1 tumor model at different doses.

[0075] **FIG. 56** shows graphs illustrating both lyophilized AXAL and clinical AXAL prolong animal survival in the TC-1 tumor model at different doses.

[0076] **FIG. 57** shows a graph illustrating reconstitution time of WP7 cycle 3 compared to cycle 1 and cycle 2.

[0077] **FIGS. 58A-B** show graphs illustrating MFI analysis at Tliq, Tlyo, and after storage for 24 and 72 hours at 30°C of A0085 (A) and A1300 (B).

[0078] **FIGS. 58C-D** show graphs illustrating MFI analysis at Tliq, Tlyo, and after storage for 24 and 72 hours at 30°C of B0085 (C) and B1300 (D).

[0079] **FIGS. 59A-B** show graphs illustrating RRM results for **A.** A0085 (200 fold dilution) and **B.** A1300 (5,000-fold dilution).

[0080] **FIGS. 59C-D** show graphs illustrating RRM results for **C.** B0085 (200 fold dilution) and **D.** B1300 (5,000-fold dilution).

[0081] **FIGS. 60A-B** show graphs illustrating negatively buoyant particle distribution for A0085 (200 fold dilution) (A) and A1300 (5,000-fold dilution) (B).

[0082] **FIGS. 60C-D** show graphs illustrating negatively buoyant particle distribution for B0085 (200 fold dilution) (C) and B1300 (5,000-fold dilution) (D).

[0083] **FIG. 61** shows a graph illustrating Karl-Fischer titration analysis of five vials at Tlyo of A0085, A1300, B0085, and B1300.

[0084] **FIG. 62** shows a graph illustrating results of VCC assay performed at Tliq, Tlyo and after storage for 24h, 48h, and 72h at 30°C. Front vials were stored at –20 °C for 7 days before analysis.

[0085] **FIG. 63A** shows a scatterplot illustrating VCC after WP7 Cycle 3 on accelerated stability.

[0086] **FIG. 63B** shows a scatterplot illustrating % live after WP7 Cycle 3 on accelerated stability.

[0087] **FIG. 64** shows a scatterplot illustrating impact on VCC of repeated freeze/thaw cycles on BDS (1L Fill/1L LDPE Bag) at various temperatures and VCC levels.

[0088] **FIG. 65** shows a scatterplot illustrating impact on % live of repeated freeze/thaw cycles on BDS (1L Fill/1L LDPE Bag) at various temperatures and VCC levels.

DEFINITIONS

[0089] The terms "protein," "polypeptide," and "peptide," used interchangeably herein, refer to polymeric forms of amino acids of any length, including coded and non-coded amino acids and chemically or biochemically modified or derivatized amino acids. The terms include polymers that have been modified, such as polypeptides having modified peptide backbones.

[0090] Proteins are said to have an "N-terminus" and a "C-terminus." The term "N-terminus" relates to the start of a protein or polypeptide, terminated by an amino acid with a free amine group (-NH2). The term "C-terminus" relates to the end of an amino acid chain (protein or polypeptide), terminated by a free carboxyl group (-COOH).

[0091] The term "fusion protein" refers to a protein comprising two or more peptides linked together by peptide bonds or other chemical bonds. The peptides can be linked together directly by a peptide or other chemical bond. For example, a chimeric molecule can be recombinantly expressed as a single-chain fusion protein. Alternatively, the peptides can be linked together by a "linker" such as one or more amino acids or another suitable linker between the two or more peptides.

[0092] The terms "nucleic acid" and "polynucleotide," used interchangeably herein, refer to polymeric forms of nucleotides of any length, including ribonucleotides, deoxyribonucleotides, or analogs or modified versions thereof. They include single-, double-, and multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, and polymers comprising purine bases, pyrimidine bases, or other natural, chemically modified, biochemically modified, non-natural, or derivatized nucleotide bases.

[0093] Nucleic acids are said to have "5′ ends" and "3′ ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5′ phosphate of one mononucleotide pentose ring is attached to the 3′ oxygen of its neighbor in one direction via a phosphodiester linkage. An end of an oligonucleotide is referred to as the "5′ end" if its 5′ phosphate is not linked to the 3′ oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is

referred to as the "3′ end" if its 3′ oxygen is not linked to a 5′ phosphate of another mononucleotide pentose ring. A nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5′ and 3′ ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5′ of the "downstream" or 3′ elements.

[0094] "Codon optimization" refers to a process of modifying a nucleic acid sequence for enhanced expression in particular host cells by replacing at least one codon of the native sequence with a codon that is more frequently or most frequently used in the genes of the host cell while maintaining the native amino acid sequence. For example, a polynucleotide encoding a fusion polypeptide can be modified to substitute codons having a higher frequency of usage in a given *Listeria* cell or any other host cell as compared to the naturally occurring nucleic acid sequence. Codon usage tables are readily available, for example, at the "Codon Usage Database." The optimal codons utilized by *L. monocytogenes* for each amino acid are shown US 2007/0207170, herein incorporated by reference in its entirety for all purposes. These tables can be adapted in a number of ways. *See* Nakamura *et al.* (2000) *Nucleic Acids Research* 28:292, herein incorporated by reference in its entirety for all purposes. Computer algorithms for codon optimization of a particular sequence for expression in a particular host are also available (*see, e.g.*, Gene Forge).

[0095] The term "plasmid" or "vector" includes any known delivery vector including a bacterial delivery vector, a viral vector delivery vector, a peptide immunotherapy delivery vector, a DNA immunotherapy delivery vector, an episomal plasmid, an integrative plasmid, or a phage vector. The term "vector" refers to a construct which is capable of delivering, and, optionally, expressing, one or more fusion polypeptides in a host cell.

[0096] The term "episomal plasmid" or "extrachromosomal plasmid" refers to a nucleic acid vector that is physically separate from chromosomal DNA (i.e., episomal or extrachromosomal and does not integrated into a host cell's genome) and replicates independently of chromosomal DNA. A plasmid may be linear or circular, and it may be single-stranded or double-stranded. Episomal plasmids may optionally persist in multiple copies in a host cell's cytoplasm (e.g., *Listeria*), resulting in amplification of any genes of interest within the episomal plasmid.

[0097] The term "genomically integrated" refers to a nucleic acid that has been introduced into a cell such that the nucleotide sequence integrates into the genome of the cell and is capable

of being inherited by progeny thereof. Any protocol may be used for the stable incorporation of a nucleic acid into the genome of a cell.

[0098] The term "stably maintained" refers to maintenance of a nucleic acid molecule or plasmid in the absence of selection (e.g., antibiotic selection) for at least 10 generations without detectable loss. For example, the period can be at least 15 generations, 20 generations, at least 25 generations, at least 30 generations, at least 40 generations, at least 50 generations, at least 60 generations, at least 80 generations, at least 100 generations, at least 150 generations, at least 200 generations, at least 300 generations, or at least 500 generations. Stably maintained can refer to a nucleic acid molecule or plasmid being maintained stably in cells *in vitro* (e.g., in culture), being maintained stably *in vivo*, or both.

[0099] An "open reading frame" or "ORF" is a portion of a DNA which contains a sequence of bases that could potentially encode a protein. As an example, an ORF can be located between the start-code sequence (initiation codon) and the stop-codon sequence (termination codon) of a gene.

[00100] A "promoter" is a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular polynucleotide sequence. A promoter may additionally comprise other regions which influence the transcription initiation rate. The promoter sequences disclosed herein modulate transcription of an operably linked polynucleotide. A promoter can be active in one or more of the cell types disclosed herein (e.g., a eukaryotic cell, a non-human mammalian cell, a human cell, a rodent cell, a pluripotent cell, a one-cell stage embryo, a differentiated cell, or a combination thereof). A promoter can be, for example, a constitutively active promoter, a conditional promoter, an inducible promoter, a temporally restricted promoter (e.g., a developmentally regulated promoter), or a spatially restricted promoter (e.g., a cell-specific or tissue-specific promoter). Examples of promoters can be found, for example, in WO 2013/176772, herein incorporated by reference in its entirety.

[00101] "Operable linkage" or being "operably linked" refers to the juxtaposition of two or more components (e.g., a promoter and another sequence element) such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. For example, a promoter can be operably linked to a coding sequence if the promoter controls the level of transcription of the

coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. Operable linkage can include such sequences being contiguous with each other or acting in trans (e.g., a regulatory sequence can act at a distance to control transcription of the coding sequence).

[00102] "Sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well-known. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

[00103] "Percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences (greatest number of perfectly matched residues) over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Unless otherwise specified (e.g., the shorter sequence includes a linked heterologous sequence), the comparison window is the full length of the shorter of the two sequences being compared.

[00104] Unless otherwise stated, sequence identity/similarity values refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. "Equivalent program" includes any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

[00105] The term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, or leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, or between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine, or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, or methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue. Typical amino acid categorizations are summarized in **Table 1** below.

[00106] Table 1. Amino Acid Categorizations.

Alanine	Ala	A	Nonpolar	Neutral	1.8
Arginine	Arg	R	Polar	Positive	–4.5
Asparagine	Asn	N	Polar	Neutral	–3.5
Aspartic acid	Asp	D	Polar	Negative	–3.5
Cysteine	Cys	C	Nonpolar	Neutral	2.5
Glutamic acid	Glu	E	Polar	Negative	–3.5
Glutamine	Gln	Q	Polar	Neutral	–3.5
Glycine	Gly	G	Nonpolar	Neutral	–0.4
Histidine	His	H	Polar	Positive	–3.2
Isoleucine	Ile	I	Nonpolar	Neutral	4.5
Leucine	Leu	L	Nonpolar	Neutral	3.8
Lysine	Lys	K	Polar	Positive	–3.9
Methionine	Met	M	Nonpolar	Neutral	1.9
Phenylalanine	Phe	F	Nonpolar	Neutral	2.8
Proline	Pro	P	Nonpolar	Neutral	–1.6
Serine	Ser	S	Polar	Neutral	–0.8
Threonine	Thr	T	Polar	Neutral	–0.7
Tryptophan	Trp	W	Nonpolar	Neutral	–0.9
Tyrosine	Tyr	Y	Polar	Neutral	–1.3
Valine	Val	V	Nonpolar	Neutral	4.2

[00107] A "homologous" sequence (e.g., nucleic acid sequence) refers to a sequence that is either identical or substantially similar to a known reference sequence, such that it is, for example, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the known reference sequence.

[00108] The term "wild type" refers to entities having a structure and/or activity as found in a normal (as contrasted with mutant, diseased, altered, or so forth) state or context. Wild type gene and polypeptides often exist in multiple different forms (e.g., alleles).

[00109] The term "isolated" with respect to proteins and nucleic acid refers to proteins and nucleic acids that are relatively purified with respect to other bacterial, viral or cellular components that may normally be present *in situ*, up to and including a substantially pure preparation of the protein and the polynucleotide. The term "isolated" also includes proteins and nucleic acids that have no naturally occurring counterpart, have been chemically synthesized and are thus substantially uncontaminated by other proteins or nucleic acids, or has been separated or

purified from most other cellular components with which they are naturally accompanied (e.g., other cellular proteins, polynucleotides, or cellular components).

[00110] "Exogenous" or "heterologous" molecules or sequences are molecules or sequences that are not normally expressed in a cell or are not normally present in a cell in that form. Normal presence includes presence with respect to the particular developmental stage and environmental conditions of the cell. An exogenous or heterologous molecule or sequence, for example, can include a mutated version of a corresponding endogenous sequence within the cell or can include a sequence corresponding to an endogenous sequence within the cell but in a different form (i.e., not within a chromosome). An exogenous or heterologous molecule or sequence in a particular cell can also be a molecule or sequence derived from a different species than a reference species of the cell or from a different organism within the same species. For example, in the case of a *Listeria* strain expressing a heterologous polypeptide, the heterologous polypeptide could be a polypeptide that is not native or endogenous to the *Listeria* strain, that is not normally expressed by the *Listeria* strain, from a source other than the *Listeria* strain, derived from a different organism within the same species.

[00111] In contrast, "endogenous" molecules or sequences or "native" molecules or sequences are molecules or sequences that are normally present in that form in a particular cell at a particular developmental stage under particular environmental conditions.

[00112] The term "variant" refers to an amino acid or nucleic acid sequence (or an organism or tissue) that is different from the majority of the population but is still sufficiently similar to the common mode to be considered to be one of them (e.g., splice variants).

[00113] The term "isoform" refers to a version of a molecule (e.g., a protein) with only slight differences compared to another isoform, or version (e.g., of the same protein). For example, protein isoforms may be produced from different but related genes, they may arise from the same gene by alternative splicing, or they may arise from single nucleotide polymorphisms.

[00114] The term "fragment" when referring to a protein means a protein that is shorter or has fewer amino acids than the full length protein. The term "fragment" when referring to a nucleic acid means a nucleic acid that is shorter or has fewer nucleotides than the full length nucleic acid. A fragment can be, for example, an N-terminal fragment (i.e., removal of a portion of the C-terminal end of the protein), a C-terminal fragment (i.e., removal of a portion of the N-

terminal end of the protein), or an internal fragment. A fragment can also be, for example, a functional fragment or an immunogenic fragment.

[00115] The term "analog" when referring to a protein means a protein that differs from a naturally occurring protein by conservative amino acid differences, by modifications which do not affect amino acid sequence, or by both.

[00116] The term "functional" refers to the innate ability of a protein or nucleic acid (or a fragment, isoform, or variant thereof) to exhibit a biological activity or function. Such biological activities or functions can include, for example, the ability to elicit an immune response when administered to a subject. Such biological activities or functions can also include, for example, binding to an interaction partner. In the case of functional fragments, isoforms, or variants, these biological functions may in fact be changed (e.g., with respect to their specificity or selectivity), but with retention of the basic biological function.

[00117] The terms "immunogenicity" or "immunogenic" refer to the innate ability of a molecule (e.g., a protein, a nucleic acid, an antigen, or an organism) to elicit an immune response in a subject when administered to the subject. Immunogenicity can be measured, for example, by a greater number of antibodies to the molecule, a greater diversity of antibodies to the molecule, a greater number of T-cells specific for the molecule, a greater cytotoxic or helper T-cell response to the molecule, and the like.

[00118] The term "antigen" is used herein to refer to a substance that, when placed in contact with a subject or organism (e.g., when present in or when detected by the subject or organism), results in a detectable immune response from the subject or organism. An antigen may be, for example, a lipid, a protein, a carbohydrate, a nucleic acid, or combinations and variations thereof. For example, an "antigenic peptide" refers to a peptide that leads to the mounting of an immune response in a subject or organism when present in or detected by the subject or organism. For example, such an "antigenic peptide" may encompass proteins that are loaded onto and presented on MHC class I and/or class II molecules on a host cell's surface and can be recognized or detected by an immune cell of the host, thereby leading to the mounting of an immune response against the protein. Such an immune response may also extend to other cells within the host, such as diseased cells (e.g., tumor or cancer cells) that express the same protein.

[00119] The term "epitope" refers to a site on an antigen that is recognized by the immune system (e.g., to which an antibody binds). An epitope can be formed from contiguous amino

acids or noncontiguous amino acids juxtaposed by tertiary folding of one or more proteins. Epitopes formed from contiguous amino acids (also known as linear epitopes) are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding (also known as conformational epitopes) are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. *See, e.g.*, Epitope Mapping Protocols, in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed. (1996), herein incorporated by reference in its entirety for all purposes.

[00120] The term "mutation" refers to the any change of the structure of a gene or a protein. For example, a mutation can result from a deletion, an insertion, a substitution, or a rearrangement of chromosome or a protein. An "insertion" changes the number of nucleotides in a gene or the number of amino acids in a protein by adding one or more additional nucleotides or amino acids. A "deletion" changes the number of nucleotides in a gene or the number of amino acids in a protein by reducing one or more additional nucleotides or amino acids.

[00121] A "frameshift" mutation in DNA occurs when the addition or loss of nucleotides changes a gene's reading frame. A reading frame consists of groups of 3 bases that each code for one amino acid. A frameshift mutation shifts the grouping of these bases and changes the code for amino acids. The resulting protein is usually nonfunctional. Insertions and deletions can each be frameshift mutations.

[00122] A "missense" mutation or substitution refers to a change in one amino acid of a protein or a point mutation in a single nucleotide resulting in a change in an encoded amino acid. A point mutation in a single nucleotide that results in a change in one amino acid is a "nonsynonymous" substitution in the DNA sequence. Nonsynonymous substitutions can also result in a "nonsense" mutation in which a codon is changed to a premature stop codon that results in truncation of the resulting protein. In contrast, a "synonymous" mutation in a DNA is one that does not alter the amino acid sequence of a protein (due to codon degeneracy).

[00123] The term "somatic mutation" includes genetic alterations acquired by a cell other than a germ cell (e.g., sperm or egg). Such mutations can be passed on to progeny of the mutated cell in the course of cell division but are not inheritable. In contrast, a germinal mutation occurs in the germ line and can be passed on to the next generation of offspring.

[00124] The term "*in vitro*" refers to artificial environments and to processes or reactions that occur within an artificial environment (e.g., a test tube).

[00125] The term "*in vivo*" refers to natural environments (e.g., a cell or organism or body) and to processes or reactions that occur within a natural environment.

[00126] The term "frozen state glass transition temperature" (Tg') refers to the following. When heated, solutions of sugar glasses undergo a second-order transition from a rigid state to a viscoelastic rubbery state. The temperature at which the vitreous transformation occurs is the glass-transition temperature in the frozen state.

[00127] The term "solid state glass transition temperature" (Tg) refers to the following. Similar to Tg', this is the temperature at which the freeze-dried glassy solid transforms to a viscoelastic rubbery state.

[00128] The term "collapse temperature" (Tc) refers to the maximum temperature that the product can withstand during primary drying without losing its physical structure.

[00129] The term "drug substance" (DS) refers to an active ingredient. It refers to any component of a drug product intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or other animals. Active ingredients include those components of the product that may undergo chemical change during the manufacture of the drug product and be present in the drug product in a modified form intended to furnish the specified activity or effect. For example, *Lm* (e.g., ADXS-HPV or ADXS-HER2) is considered a drug substance.

[00130] The term "bulk drug substance" (BDS) refers to any substance that is represented for use in a drug and that, when used in the manufacturing, processing, or packaging of a drug, becomes an active ingredient or a finished dosage form of the drug, but the term does not include intermediates used in the synthesis of such substances.

[00131] The term "drug product" (DP) refers to a finished dosage form, for example, a tablet, capsule or solution that contains an active pharmaceutical ingredient, generally, but not necessarily, in association with inactive ingredients. For example, lyophilized *Lm* (e.g., ADXS-HPV or ADXS-HER2) is considered a drug product.

[00132] Compositions or methods "comprising" or "including" one or more recited elements may include other elements not specifically recited. For example, a composition that

"comprises" or "includes" a protein may contain the protein alone or in combination with other ingredients.

[00133] Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range.

[00134] Unless otherwise apparent from the context, the term "about" encompasses values within a standard margin of error of measurement (e.g., SEM) of a stated value or variations ± 0.5%, 1%, 5%, or 10% from a specified value.

[00135] The singular forms of the articles "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an antigen" or "at least one antigen" can include a plurality of antigens, including mixtures thereof.

[00136] Statistically significant means $p \leq 0.05$.

DETAILED DESCRIPTION

I. Overview

[00137] Disclosed herein are compositions and methods directed to a stable lyophilized pharmaceutical formulation prepared by lyophilizing an aqueous formulation comprising a bacteria or *Listeria* strain, such as *Listeria monocytogenes*. In some embodiments, the lyophilized formulation is stable at 4°C or −20°C for at least 6 months, at least 1 year, or at least 2 years. In some embodiments, the lyophilized formulation is suitable for parenteral administration such as intravenous injection.

[00138] The frozen liquid formulations currently used for therapeutics comprising, for example, *L. monocytogenes*, are stored and shipped at −80°C. The low temperature presents supply chain challenges with both shipping and storage of the material at clinical sites, particularly in countries in South America and Africa. Therefore, it is desirable to have a refrigerated or −20°C supply chain. Through optimization of the manufacturing process as described herein, it is possible to generate a stable drug product that is able to be maintained at higher temperatures. Counterintuitively, the working examples described herein show that higher residual moistures (e.g., higher than normal targeted residual moisture levels such as about 2.5%, 3.0%, or 3.5% in one embodiment) improved the stability of the lyophilized product. Similarly, counterintuitively, higher shelf temperatures during the primary drying step (e.g., about −17°C to about −19°C or about −18°C in one embodiment, which is well above the Tg)

improved the stability of the lyophilized product. In addition, preconditioning of the cells prior to lyophilization through heat shock improved the stability of the lyophilized product. In addition, use of a higher concentration of viable bacteria (viable cell count, or VCC) results in an improvement in the stability of the lyophilized drug product relative to lower VCC. In addition, counterintuitively, thawing of frozen drug substance at about 37°C prior to lyophilization improved stability of the lyophilized drug product relative to thawing at room temperature or 2-8°C. These process enhancements improve stability at higher temperatures compared to a liquid frozen formulation allowing for a higher temperature supply chain. This allows for a more manageable supply chain and distribution to countries were –80°C storage is not feasible.

[00139] The process of inducing a stress response within the *L. monocytogenes* cells through a temperature shift prior to lyophilization in addition to optimization of the residual moisture in the lyophilized cake (e.g., using higher than normal targeted residual moisture levels such as about 3.5% in one embodiment, which can be achieved by altering the secondary drying temperature and optionally the secondary drying time) improve stability of the lyophilized drug product at higher temperatures. Similarly, use of formulations comprising a phosphate buffer and lower than normal levels of sucrose (e.g., about 2.5% w/v sucrose in one embodiment) and using high primary drying step temperatures (e.g., about –18°C in one embodiment) improve stability of the lyophilized drug product at higher temperatures, including –20°C, 2-8°C, and even room temperature (about 20°C to about 25°C, or about 20°C, about 23°C, or about 25°C). These process enhancements improve stability at higher temperatures compared to a liquid frozen formulation allowing for a higher temperature supply chain. This allows for a more manageable supply chain and distribution to countries were –80°C storage is not feasible.

II. Lyophilization of Bacteria or Listeria

[00140] Lyophilization can be divided in three steps: freezing, primary drying, and secondary drying. As water freezes in the first step, the dissolved components in the formulation remain in the residual liquid (the freeze-concentrate). At the point of maximal ice formation, the freeze-concentrate solidifies between the ice crystals that make up the lattice. Under appropriate lyophilization conditions, the ice is removed by sublimation during primary drying, leaving the remaining freeze-concentrate in the same physical and chemical structure as when the ice was present. Residual water in the freeze-concentrate is removed in the secondary drying step.

[00141] Lyophilization involves manipulating the temperature and pressure of the solution so that the phase of the solvent can move directly from the frozen state to the gaseous state without moving through the liquid phase/state. This is achieved by cooling the solution and lowering the pressure to below the triple point of water. This allows for the removal of the solvent from the product without subjecting the product to intense heat. During the freezing stage, the formulation is cooled. Pure crystalline ice forms from the liquid, thereby resulting in a freeze concentration of the remainder of the liquid to a more viscous state that inhibits further crystallization. Ultimately, this highly concentrated and viscous solution solidifies, yielding an amorphous, crystalline, or combined amorphous–crystalline phase. During the primary drying stage, the ice formed during freezing is removed by sublimation at sub-ambient temperatures under vacuum. Throughout this stage, the product is maintained in the solid state below the collapse temperature of the product in order to dry the product with retention of the structure established in the freezing step. The collapse temperature is the glass transition temperature (Tg') in the case of amorphous products or the eutectic temperature (Te) for crystalline products. During the secondary drying stage, the relatively small amount of bound water remaining in the matrix is removed by desorption. During this stage, the temperature of the shelf and product are increased to promote adequate desorption rates and achieve the desired residual moisture.

[00142] The target profile for a lyophilized drug product is one that produces a well-defined cake at a target residual moisture that is stable at either 2-8°C or –20°C and retains the same potency and biological activity as the liquid-frozen formulation. Protection strategies that may enhance bacterial viability during freeze drying include, for example, adding excipients to the drying medium, controlling the process parameters, pre-stressing the bacterial sample prior to freeze drying, and changing the fermentation conditions of the bacteria. However, the efficiency of these strategies is strain-dependent, because the intrinsic tolerance to the drying process varies also from strain to strain. Even in highly related bacteria strains, one strain may be much more resistant to the freeze-drying process than the other. This strain dependency makes it difficult to draw general conclusions and guidelines.

[00143] Provided herein are methods for producing a lyophilized composition comprising a bacteria or *Listeria* strain. Such methods can comprise providing a composition comprising a bacteria or *Listeria* strain in a formulation comprising a buffer, cooling the composition in a freezing step, exposing the cooled composition to a vacuum and a first increased temperature in a

primary drying step, and exposing the composition from the primary drying step to a vacuum and a second increased temperature in a secondary drying step, whereby the lyophilized composition is produced.

[00144] In some such methods, the bacteria or *Listeria* strain used in the composition is a frozen *Listeria* strain that is thawed prior to the freezing step. Examples of such preconditioning steps are described in more detail elsewhere herein. In a specific example, the frozen bacteria or *Listeria* strain can be thawed at a temperature of about 2°C to about 37°C, about 20°C to about 37°C, about 23°C to about 37°C, about 25°C to about 37°C, about 32°C to about 37°C, or about 37°C. Optionally, the thawing is for no more than about 8 hours. Optionally, the thawed bacteria or *Listeria* strain is held at temperature of between about 2°C and about 8°C for no more than about 24 hours. In a specific example, the concentration of the bacteria or *Listeria* strain being thawed can be between about 1x10E9 and about 1x10E10 colony forming units (CFU) per milliliter.

[00145] In some such methods, the formulation comprises a buffer and sucrose. For example, the formulation buffer can comprise about 1% to about 5% w/v sucrose, about 2% to about 3% w/v sucrose, or about 2.5% w/v sucrose. Optionally, the formulation does not comprise other excipients such as trehalose, monosodium glutamate (MSG), or recombinant human serum albumin (rHSA).

[00146] In some such methods, the formulation comprises about 1x10E9 to about 1x10E10 colony forming units (CFU) of bacteria or *Listeria* per milliliter.

[00147] In some such methods, the holding temperature in the primary drying step is between about −10°C and about −30°C, between about −12°C and about −22°C, between about −17°C and about −19°C, or about −18°C.

[00148] In some such methods, the residual moisture in the lyophilized composition is at least about 2.5%, at least about 3%, or at least about 3.5%. In some such methods, the residual moisture is between about 1% and about 5% or between about 2% and about 4%.

[00149] In some such methods, the lyophilized composition shows at least about 60%, 70%, 80%, or 90% viability after storage at between about −20°C and about 4°C or after storage at about −20°C or about 4°C for about 6 months, 12 months, 18 months, or 24 months.

[00150] Some such methods comprise: (a) providing a composition comprising a bacteria or *Listeria* strain in a formulation comprising a buffer and sucrose; (b) cooling the composition

provided in step (a) at a holding temperature between about –32°C and about –80°C in a freezing step; (c) exposing the composition produced by step (b) to a vacuum at a holding temperature between about −10°C and about −30°C in a primary drying step; and (d) exposing the composition produced by step (c) to a vacuum at a holding temperature between about –5°C and about 25°C in a secondary drying step.

[00151] Additional embodiments for the preconditioning of cells, the formulations, the freezing step, the primary drying step, the secondary drying step, and the lyophilized product are provided below.

A. Pre-Conditioning of Bacteria or *Listeria*

[00152] A culture of a bacteria or *Listeria* strain that is used in a lyophilization method disclosed herein can be from a frozen stock, from a starter culture, or from a colony (e.g., freshly cultured bacteria or *Listeria*).

[00153] Methods are provided herein for preparing a frozen bacteria or *Listeria* strain for lyophilization, comprising thawing the frozen bacteria or *Listeria* strain. If the bacteria or *Listeria* strain is from a frozen stock, it can be thawed by any means. Temperature and the time for thawing can impact stability. Identifying appropriate conditions for thawing frozen drug substance allows freezing and holding of the drug substance prior to lyophilization. Ensuring high-quality healthy cells coming out of thaw ensures that the resulting lyophilized drug product is also of sufficient quality. In one example, it can be thawed at about –4°C, about 2-8°C, or about 4°C and incubated, for example, for about 0.5, 1, 2, 3, 4, or more hours. In another example, it can be thawed at about 37°C and incubated, for example, for about 0.5, 1, 2, 3, 4, or more hours.

[00154] In one example, the frozen bacteria or *Listeria* strain can be thawed a temperature between about 4°C and about 37°C, about 10°C and about 37°C, about 15°C and about 37°C, about 20°C and about 37°C, about 23°C and about 37°C, about 25°C and about 37°C, about 25°C and about 37°C, about 30°C and about 37°C, about 32°C and about 37°C, about 32°C and about 42°C, about 34°C and about 40°C, about 35°C and about 39°C, about 36°C and 38°C, or about 37°C.

[00155] The frozen bacteria or *Listeria* strain can be thawed, for example, for about 0.5, 1, 2, 3, 4, 5, 6, 7, or 8 hours, for between about 0.5 and about 8 hours, between about 1 and about 8

hours, between about 2 and about 8 hours, between about 3 and about 8 hours, between about 4 and about 8 hours, between about 5 and about 8 hours, between about 6 and about 8 hours, or between about 7 and about 8 hours. Alternatively, the frozen bacteria or *Listeria* strain can be thawed, for example, for no more than about 0.5, 1, 2, 3, 4, 5, 6, 7, or 8 hours.

[00156] The frozen bacteria or *Listeria* strain being thawed can be in a bacteria or *Listeria* lyophilization formulation or can be thawed in a bacteria or *Listeria* lyophilization formulation. Such bacteria or *Listeria* lyophilization formulations are disclosed in more detail elsewhere herein.

[00157] The frozen bacteria or *Listeria* strain can be held at a temperature after thawing. For example, the frozen bacteria or *Listeria* strain can be held at a temperature of between about 2°C to about 8°C after thawing. The frozen bacteria or *Listeria* strain can be held, for example, for about 0.5 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 or between about 0.5 and about 24 hours, between about 1 and about 24 hours, between about 2 and about 24 hours, between about 5 and about 24 hours, between about 10 and about 24 hours, between about 12 and about 24 hours. Alternatively, the frozen bacteria or *Listeria* strain can be held, for example, for no more than about 0.5 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. In a specific example, the frozen bacteria or *Listeria* strain is thawed at a temperature of about 37°C for no more than about 8 hours and is held at temperature of between about 2°C and about 8°C for no more than about 24 hours.

[00158] The concentration of the bacteria or *Listeria* strain being thawed can be any suitable concentration. For example, the concentration can be between about 1x10E9 and about 1x10E10 colony forming units (CFU) per milliliter.

[00159] The culture used for lyophilization can be at any growth phase. The culture can be, for example, at mid-log growth phase, at approximately mid-log growth phase, or at another growth phase.

[00160] The nutrient medium utilized for growing a culture of a bacteria or *Listeria* strain can be any suitable nutrient medium. Examples of suitable media include, for example, Luria broth (LB; Luria-Bertani broth); Terrific Broth (TB); a modified, animal-product-free Terrific Broth; or a defined medium. The bacteria or *Listeria* strain can be cultured by any known means of growing bacteria. For example, the step of growing can be performed with a shake flask (such as

a baffled shake flask), a batch fermenter, a stirred tank or flask, an airlift fermenter, a fed batch, a continuous cell reactor, an immobilized cell reactor, or any other means of growing bacteria.

[00161] Optionally, a constant pH is maintained during growth of the culture (e.g. in a batch fermenter). For example, the pH can be maintained at about 6.0, at about 6.5, at about 7.0, at about 7.5, or about 8.0. Likewise, the pH can be, for example, from about 6.5 to about 7.5, from about 6.0 to about 8.0, from about 6.0 to about 7.0, from about 6.0 to about 7.0, or from about 6.5 to about 7.5. Alternatively, immediately after harvesting the cells from the bioreactor, the pH can be dropped by the addition of acid to induce a stress response, which can activate a set of genes that may better prepare the cells for lyophilization.

[00162] Optionally, a constant temperature can be maintained during growth of the culture. For example, the temperature can be maintained at about 37°C. Alternatively, the temperature can be maintained at about 25°C, about 27°C, about 28°C, about 30°C, about 32°C, about 34°C, about 35°C, about 36°C, about 38°C, or about 39°C. Alternatively, immediately after harvesting the cells from the bioreactor, the temperature can be dropped by placing the cells in an ice bath (e.g., about 0°C or about 4°C) to induce a stress response, which can activate a set of genes that may better prepare the cells for lyophilization.

[00163] Optionally, a constant dissolved oxygen concentration can be maintained during growth of the culture. For example, the dissolved oxygen concentration can be maintained at 20% of saturation, 15% of saturation, 16% of saturation, 18% of saturation, 22% of saturation, 25% of saturation, 30% of saturation, 35% of saturation, 40% of saturation, 45% of saturation, 50% of saturation, 55% of saturation, 60% of saturation, 65% of saturation, 70% of saturation, 75% of saturation, 80% of saturation, 85% of saturation, 90% of saturation, 95% of saturation, 100% of saturation, or near 100% of saturation.

[00164] The bacteria strain or *Listeria* strain can optionally be passaged through an animal host prior to lyophilization. Such passaging can maximize efficacy of the *Listeria* strain as a vaccine vector, can stabilize the immunogenicity of the *Listeria* strain, can stabilize the virulence of the *Listeria* strain, can increase the immunogenicity of the *Listeria* strain, can increase the virulence of the *Listeria* strain, can remove unstable sub-strains of the *Listeria* strain, or can reduce the prevalence of unstable sub-strains of the *Listeria* strain. Methods for passaging a *Listeria* strain through an animal host are well-known and are described, for example, in US 2006/0233835, herein incorporated by reference in its entirety for all purposes.

B. Bacteria or *Listeria* Lyophilization Formulations

[00165] Prior to the lyophilization, the bacteria or *Listeria* strain can be provided in a suspension (formulation) comprising a buffer and an excipient. The design of a lyophilized formulation can depend on the requirements of the active pharmaceutical ingredient and the intended route of administration. A formulation may consist of a buffer and one or more excipients that perform one or more functions. Such excipients can be, for example, pH adjusters, bulking agents (e.g., sucrose, mannitol, maltose, trehalose, dextrose, and lactose), stabilizers such as cryoprotectants (e.g., PEG) and lyoprotectants (e.g., disaccharides), or tonicity modifiers (e.g., NaCl, mannitol, sucrose, glycine, and glycerol).

[00166] The buffer can be any suitable buffer. Buffers can stabilize pH in the formulation. For example, the buffer can be a phosphate buffer, a Tris buffer, a histidine buffer, a citrate buffer, or a MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer. In a specific example, the buffer is a phosphate buffer. Phosphate buffers are often avoided in the development of lyophilized formulations because phosphate buffers, such as sodium phosphate, can undergo drastic pH changes during freezing. Because of this, low concentrations of buffers that undergo minimal pH changes during freezing, such as Tris, citrate, and histidine buffers are often used. However, as shown elsewhere herein, suitable viability levels of *Listeria monocytogenes* are achieved using phosphate buffers. In some such buffers, the concentration of KH_2PO_4 (anhydrous) is between about 0.1-0.3 g/L, 0.12-0.28 g/L, 0.14-0.26 g/L, 0.16-0.24 g/L, 0.18-0.22 g/L, 0.19-0.21 g/L, or 0.2 g/L. In some such buffers, the concentration of Na_2HPO_4 (anhydrous) is between about 1.0-1.3 g/L, 1.02-1.28 g/L, 1.04-1.26 g/L, 1.06-1.24 g/L, 1.08-1.22 g/L, 1.1-1.2 g/L, 1.12-1.18 g/L, 1.14-1.16 g/L, or 1.15 g/L. Some such buffers are about 5-20, 6-18, 7-16, 8-14, 9-12, 9-11, or 10 mM. Some such buffers have a pH of about 6.8-7.6, 6.9-7.5, 7.0-7.4, 7.1-7.3, or 7.2. As one example, the phosphate buffer can have between about 0.19-0.21 g/L (e.g., 0.2 g/L) of KH_2PO_4 (anhydrous), between about 1.14-1.16 g/L (e.g., 1.15 g/L) of Na_2HPO_4 (anhydrous), and can have a pH of about 7.1-7.3 (e.g., 7.2).

[00167] Excipients such as cryoprotectants and lyoprotectants can be added to the formulation to protect the bacteria or *Listeria* strain during the lyophilization process. Cryoprotectants are water-soluble chemicals that lower the melting point of water. As ice crystals are formed, bacterial cells are compressed in the unfrozen fraction. Adding cryoprotectants can enlarge the

unfrozen section, giving more space to the bacterial cells, which can lead to less cellular damage by mechanical stress or osmotic stress. Lyoprotectants can protect bacterial cells during the drying steps when water is removed. Some sugars, such as sucrose and trehalose, can act as both cryoprotectants and lyoprotectants. Use of skim milk can also provide protective effects. Other examples of excipients include glucose, maltose, lactose, mannitol, glycine, glycerol, sodium chloride, yeast extract, dextran, dextrose, polydextrose, monosodium glutamate, maltodextrin, antioxidants (e.g., ascorbic acid), saccharides, disaccharides, sugars, and others. In one example, excipients used in a formulation include various combinations of sucrose, trehalose, monosodium glutamate (MSG), recombinant human serum albumin (rHSA), and amino acid mix. In a specific example, the excipients comprise, consist essentially of, or consist of sucrose, such as about 5% w/v (weight per volume) sucrose or about 2.5% w/v sucrose. For example, the formulation buffer can comprise about 1% to about 5% w/v sucrose, about 2% to about 3% w/v sucrose, or about 2.5% w/v sucrose.

[00168] Optionally, the excipients do not include one or more or all of trehalose, MSG, rHSA, amino acid mix, skim milk, glucose, maltose, lactose, mannitol, glycine, glycerol, sodium chloride, yeast extract, dextran, dextrose, polydextrose, monosodium glutamate, maltodextrin, ascorbic acid, saccharides other than sucrose, disaccharides other than sucrose, sugars other than sucrose, or antioxidants. Optionally, the excipients do not include one or more or all of trehalose, MSG, and rHSA.

[00169] The concentration of the bacteria or *Listeria* in the formulation can be any suitable concentration. For example, the concentration can be between about 1x10E9 and about 1x10E10 colony forming units (CFU) per milliliter.

C. Freezing Step

[00170] The first step in lyophilization is the freezing step. During this stage, the formulation is cooled. This can be accomplished, for example, in a shelf freeze dryer by reducing the temperature of the lyophilizer shelves (i.e., reducing the shelf temperature). During freezing, ice crystals are formed that can damage bacteria. The growth of the ice crystals is dependent on the freezing rate and temperature. In some embodiments, a higher freezing rate is utilized. A higher freezing rater can lead to the formation of smaller ice crystals thus reducing cellular damage as compared to a slower freezing rate. The formation of ice crystals can be detrimental to bacteria.

As water crystallizes, the solutes in the remaining unfrozen fraction concentrate, which can lead to chemical and osmotic damage. Although freezing bacteria at lower temperatures corresponds to higher freezing rates and will result in smaller ice crystals, which should limit the cellular damage, a higher freezing rate does not always corresponds with the best viability results. Optimal freezing conditions can vary depending on protectants used in the formulation and the strain of bacteria.

[00171] The holding temperature (e.g., the shelf temperature) for the freezing step can be reached by reducing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 2.0°C per minute. Alternatively, the holding temperature (e.g., the shelf temperature) for the freezing step can be reached by reducing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 1.8°C per minute, about 0.4°C to about 1.6°C per minute, about 0.6°C to about 1.4°C per minute, about 0.8°C to about 1.2°C per minute, or about 0.9°C to about 1.1°C per minute. For example, the temperature can be reduced to the freezing temperature at a rate of about 0.2°C, about 0.3°C, about 0.4°C, about 0.5°C, about 0.6°C, about 0.7°C, about 0.8°C, about 0.9°C, about 1.0°C, about 1.1°C, about 1.2°C, about 1.3°C, about 1.4°C, about 1.5°C, about 1.6°C, about 1.7°C, about 1.8°C, about 1.9°C, or about 2.0°C per minute. In a specific example, the holding temperature for the freezing step is reached by decreasing the temperature to the holding temperature at a rate of about 1°C per minute.

[00172] The freezing step can be for any suitable time for freezing the bacteria or *Listeria* strain. Likewise, the temperature can be held at the freezing temperature for any suitable time for freezing the bacteria or *Listeria* strain. For example, the freezing step can be, or the temperature can be held at the freezing temperature, for about 2 to about 6, about 2.5 to about 6, about 1 to about 6, about 1 to about 5, about 1 to about 4, about 1 to about 3, about 1 to about 2, about 1.5 to about 2.5, about 1.5 to about 5.5, about 2 to about 5, about 2.5 to about 4.5, about 3 to about 4, about 1, about 1.5, about 2, about 2.5, about 3, about 3.5, about 4, about 4.5, about 5, about 5.5, or about 6 hours. In a specific example, the freezing step can be or the temperature can be held at the freezing temperature for 3.5 hours. In another specific example, freezing step can be or the temperature can be held at the freezing temperature for 2 hours. In another specific example, freezing step can be or the temperature can be held at the freezing temperature for 1.5 hours. In another specific example, the time of the entire freezing step (e.g., ramping the

temperature to the freezing temperature and then holding at the freezing temperature) is about 3.5-4.5 hours or about 3.5 hours.

[00173] The freezing temperature (i.e., the holding temperature) can be any temperature suitable for freezing the bacteria or *Listeria* strain. In some embodiments, the freezing temperature (e.g., the shelf temperature) is such that the temperature of the formulation is below the glass transition temperature of the solution, which in the case of sucrose formulations can be, for example, about −32°C. Temperatures above this temperature may not truly freeze the solution, which may then undergo collapse during lyophilization, potentially leading to loss of viability. For example, the temperature (e.g., the shelf temperature) can be between about −49°C to about −25°C, about −47°C to about −40°C, about −45°C to about −35°C, about −10°C to about −80°C, about −15°C to about −75°C, about −20°C to about −70°C, about −25°C to about −65°C, about −30°C to about −60°C, about −35°C to about −55°C, about −40°C to about −50°C, about −41°C to about −49°C, about −42°C to about −48°C, about −43°C to about −47°C, or about −44°C to about −46°C. In a specific example, the freezing temperature can be about −45°C. In another example, the temperature can be between about −49°C to about −32°C, about −47°C to about −40°C, about −45°C to about −35°C, about −32°C to about −80°C, about −32°C to about −75°C, about −32°C to about −70°C, about −32°C to about −65°C, about −32°C to about −60°C, −49°C to about −33°C, about −33°C to about −80°C, about −33°C to about −75°C, about −33°C to about −70°C, about −33°C to about −65°C, about −33°C to about −60°C, about −35°C to about −55°C, about −40°C to about −50°C, about −41°C to about −49°C, about −42°C to about −48°C, about −43°C to about −47°C, or about −44°C to about −46°C. In one example, the freezing temperature can be about −39°C. In another example, the freezing temperature can be about −45°C. In a specific example, the holding temperature in the freezing step is between about −40°C and about −50°C (e.g., about −45°C), the freezing step comprises decreasing the temperature to the holding temperature at a rate of about 1°C per minute, and the cooling in the freezing step is from about 2 hours to about 4 hours (e.g., the freezing step comprises holding the composition at the holding temperature for about 2 hours).

D. Primary Drying Step

[00174] The second step in lyophilization is the primary drying step. In the primary drying step, exposing the composition comprising the bacteria or *Listeria* strain produced by the

freezing step to a vacuum at an increased temperature. In this step, the frozen water is removed by sublimation under vacuum.

[00175] The temperature for the primary drying step can be reached by increasing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 2.0°C per minute. Alternatively, the holding temperature (e.g., the shelf temperature) for the primary drying step can be reached by increasing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 1.8°C per minute, about 0.4°C to about 1.6°C per minute, about 0.6°C to about 1.4°C per minute, about 0.8°C to about 1.2°C per minute, or about 0.9°C to about 1.1°C per minute. For example, the temperature can be increased to the primary drying temperature at a rate of about 0.2°C, about 0.3°C, about 0.4°C, about 0.5°C, about 0.6°C, about 0.7°C, about 0.8°C, about 0.9°C, about 1.0°C, about 1.1°C, about 1.2°C, about 1.3°C, about 1.4°C, about 1.5°C, about 1.6°C, about 1.7°C, about 1.8°C, about 1.9°C, or about 2.0°C per minute. In a specific example, the holding temperature for the primary drying step is reached by increasing the temperature to the holding temperature at a rate of about 1°C per minute.

[00176] The primary drying step can be for any suitable time. Likewise, the holding temperature (e.g., the shelf temperature) can be held at the primary drying temperature for any suitable time. The temperature should be held at the primary drying temperature until primary drying is completed. This time can vary depending on the lyophilizer, the vial size, the fill volume, the number of vials, the pressure, and other variables. The end of primary drying can be determined, for example, when the product temperature rises to a value at or above the shelf temperature. It can also be determined, for example, by a pressure rise test in which the freeze drying chamber is isolated from the vacuum pump to determine how much the pressure rises due to continued water sublimation. For example, the primary drying step can be or the temperature can be held at the primary drying temperature for about 10 to about 29, about 29 to about 42, about 36, about 10 to about 80, about 10 to about 70, about 10 to about 60, about 10 to about 50, about 10 to about 40, about 10 to about 30, or about 20 to about 30 hours. In a specific example, primary drying step can be or the temperature can be held at the primary drying temperature for about 25 to about 35, about 26 to about 34, about 27 to about 33, about 28 to about 32, about 29 to about 31, or about 30 hours. In another specific example, primary drying step can be or the temperature can be held at the primary drying temperature for about 20 to about 30, about 21 to

about 30, about 22 to about 30, about 23 to about 29, about 24 to about 28, about 25 to about 27, or about 26 hours.

[00177] The primary drying step can be or the temperature can be held at the primary drying holding temperature for a time period defined as about 8 to about 20, about 9 to about 19, about 10 to about 18, about 11 to about 17, about 12 to about 16, about 13 to about 15, or about 14 hours after a probe in the lyophilizer (e.g., a probe for cold spots in the lyophilizer, such as in the center of the lyophilizer) has crossed the primary drying holding temperature or the T_s set point (e.g., of about −18°C). Alternatively, the drying step can be or the temperature can be held at the primary drying holding temperature for a time period defined as about 8 to about 20, about 9 to about 19, about 10 to about 18, about 11 to about 17, about 12 to about 16, about 13 to about 15, or about 14 hours after the composition being lyophilized (e.g., the samples of the compositions in the cold spots of the lyophilizer, or all samples of the compositions in the lyophilizer) has reached the primary drying holding temperature or the T_s set point (e.g., about −18°C). In a specific example, the primary drying step can be or the temperature can be held at the primary drying holding temperature for a time period defined as about 14 hours after a probe in the lyophilizer (e.g., a probe for cold spots in the lyophilizer, such as in the center of the lyophilizer) has crossed the primary drying holding temperature or the T_s set point (e.g., about −18°C) or after the composition being lyophilized (e.g., the samples of the compositions in the cold spots of the lyophilizer, or all samples of the compositions in the lyophilizer) has reached the primary drying holding temperature or the T_s set point (e.g., about −18°C), which can be, for example, about 30 hours.

[00178] The end of the primary drying step can be about 8 to about 20, about 9 to about 19, about 10 to about 18, about 11 to about 17, about 12 to about 16, about 13 to about 15, or about 14 hours after a probe in the lyophilizer (e.g., a probe for cold spots in the lyophilizer, such as in the center of the lyophilizer) has crossed the primary drying holding temperature or the T_s set point (e.g., about −18°C). Alternatively, the end of the primary drying step can be about 8 to about 20, about 9 to about 19, about 10 to about 18, about 11 to about 17, about 12 to about 16, about 13 to about 15, or about 14 hours after the composition being lyophilized (e.g., the samples of the compositions in the cold spots of the lyophilizer, or all samples of the compositions in the lyophilizer) has reached the primary drying holding temperature or the T_s set point (e.g., of about −18°C). In a specific example, the end of the primary drying step can be about 14 hours after a

probe in the lyophilizer (e.g., a probe for cold spots in the lyophilizer, such as in the center of the lyophilizer) has crossed the primary drying holding temperature or the T_s set point (e.g., about −18°C) or after the composition being lyophilized (e.g., the samples of the compositions in the cold spots of the lyophilizer, or all samples of the compositions in the lyophilizer) has reached the primary drying holding temperature or the T_s set point (e.g., of about −18°C), which can be, for example, about 30 hours.

[00179] The primary drying temperature (e.g., the shelf temperature or the holding temperature) can be any temperature suitable for drying the bacteria or *Listeria* strain. For example, the holding temperature can be between about 0°C to about −30°C, 0°C to about −19°C, about −5°C to about −30°C, about −10°C to about −25°C, about −15°C to about −20°C, about −17°C to about −19°C, about −12°C to about −30°C, about −12°C to about −24°C, about −12°C to about −22°C, about −14°C to about −22°C, about −15°C to about −21°C, about −16°C to about −20°C, about −17°C to about −19°C, about −18°C to about −22°C, about −30°, about −29°, about −28°, about −27°, about −26°, about −25°, about −24°, about −23°, about −22°, about −21°, about −20°, about −19°, about −18°, about −17°, about −16°, about −15°, about −14°, about −13°, about −12°, about −11°, about −10°, about −9°, about −8°, about −7°, about −6°, about −5°, about −4°, about −3°, about −2°, about −1°, or about 0°. For example, the temperature can be no more than about −30°, about −29°, about −28°, about −27°, about −26°, about −25°, about −24°, about −23°, about −22°, about −21°, about −20°, about −19°, about −18°, about −17°, about −16°, about −15°, about −14°, about −13°, about −12°, about −11°, or about −10°C. In a specific example, the primary drying temperature can be about −25°C to about −35°C, about −26°C to about −37°C, about −27°C to about −33°C, about −28°C to about −32°C, about −29°C to about −31°C, or about −30°C. In a specific example, the primary drying temperature can be about −17°C to about −27°C, about −18°C to about −26°C, about −19°C to about −25°C, about −20°C to about −24°C, about −21°C to about −23°C, or about −22°C. In another specific example, the primary drying temperature can be about −7°C to about −17°C, about −8°C to about −16°C, about −9°C to about −15°C, about −10°C to about −14°C, about −11°C to about −13°C, or about −12°C. In another specific example, the primary drying temperature can be about −13°C to about −23°C, about −14°C to about −22°C, about −15°C to about −21°C, about −16°C to about −20°C, about −17°C to about −19°C, or about −18°C. In a

specific example, the holding temperature in the primary drying step is between about −17°C to about −19°C, or about −18°C.

[00180] The pressure (vacuum conditions) can be any suitable pressure. In some cases, the pressure should be no more than 50% of vapor pressure of ice at the glass transition temperature of the formulation (e.g., about 0.270 mbar). It should also not be too low. For example, the pressure can be from about 0.140 to about 0.050, about 0.100 to about 0.060, about 0.100 to about 0.070, about 0.100 to about 0.080, about 0.099 to about 0.081, about 0.098 to about 0.082, about 0.097 to about 0.083, about 0.096 to about 0.084, about 0.095 to about 0.085, about 0.094 to about 0.086, about 0.093 to about 0.087, about 0.092 to about 0.088, about 0.091 to about 0.089, about 0.090 mbar, or about 0.120 mbar. In a specific example, the pressure is about 0.090 mbar.

[00181] In a specific example, the holding temperature in the primary drying step is between about −17°C and about −19°C (e.g., about −18°C), the primary drying step comprises increasing the temperature to the holding temperature at a rate of about 1°C per minute, and the primary drying step is from about 10 hours to about 40 hours (e.g., about 20 to about 40 hours, or about 25-35 hours, such as about 30 hours or about 32 hours).

E. Secondary Drying Step

[00182] The third step in lyophilization is the secondary drying step. In the secondary drying step, exposing the composition comprising the bacteria or *Listeria* strain produced by the primary drying step to a vacuum at an increased temperature. In this step, the unfrozen water is removed by desorption.

[00183] The temperature for the secondary drying step can be reached by increasing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 2.0°C per minute. Alternatively, the holding temperature (e.g., the shelf temperature) for the secondary drying step can be reached by increasing the temperature (e.g., the shelf temperature) at a rate of, for example, about 0.2°C to about 1.8°C per minute, about 0.2°C to about 1.6°C per minute, about 0.2°C to about 1.4°C per minute, about 0.2°C to about 1.2°C per minute, about 0.2°C to about 1.0°C per minute, about 0.2°C to about 0.8°C per minute, about 0.2°C to about 0.6°C per minute, about 0.2°C to about 0.4°C per minute. For example, the temperature can be increased to the secondary drying temperature at a rate of about 0.2°C, about 0.3°C, about 0.4°C, about

0.5°C, about 0.6°C, about 0.7°C, about 0.8°C, about 0.9°C, about 1.0°C, about 1.1°C, about 1.2°C, about 1.3°C, about 1.4°C, about 1.5°C, about 1.6°C, about 1.7°C, about 1.8°C, about 1.9°C, or about 2.0°C per minute. In a specific example, the holding temperature for the secondary drying step is reached by increasing the temperature to the holding temperature at a rate of about 0.2°C per minute.

[00184] The secondary drying step can be for any suitable time. Likewise, the temperature (e.g., the shelf temperature or the holding temperature) can be held at the secondary drying temperature for any suitable time. For example, the temperature can be held at the secondary drying temperature for any suitable time to achieve the desired residual moisture levels in the lyophilized product. For example, the secondary drying step can be or the temperature can be held at the secondary drying temperature for about 5 to about 40, about 10 to about 30, about 15 to about 25, about 2 to about 25, about 2 to about 20, about 2 to about 10, about 2 to about 4, about 1 to about 25, about 1 to about 20, about 1 to about 10, about 1 to about 9, about 1 to about 8, about 1 to about 7, about 1 to about 6, about 1 to about 5, about 1 to about 4, about 1 to about 3, about 1 to about 2, about 1.5 to about 2.5, about 2.5 to about 3.5, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2, or about 1 hours. In a specific example, secondary drying step can be or the secondary drying hold time is no more than 10 hours. In another specific example, the secondary drying hold time is no more than 6 hours. In another specific example, the temperature can be held at the secondary drying temperature for about 3 hours. In another specific example, the temperature can be held at the secondary drying temperature for about 2 hours. In one example, the secondary drying step is for about 1 hour to about 10 hours. In another example, the secondary drying step comprises holding the composition at the holding temperature for about 2 hours to about 6 hours, for about 5 hours to about 6 hours, or for about 5 hours or about 6 hours.

[00185] The secondary drying temperature (e.g., the shelf temperature or the holding temperature) can be any temperature suitable for drying the bacteria or *Listeria* strain to achieve the desired residual moisture levels in the lyophilized product. For example, the temperature can be between about 5°C to about 40°C, about 5°C to about 30°C, about 10°C to about 30°C, about 20°C to about 30°C, or about 15°C to about 25°C. In a specific example, the secondary drying temperature can be about 25°C. In another specific example, the secondary drying temperature can be about 20°C. In another specific example, the secondary drying temperature is no more

than about 20°C. In another example, the temperature can be between about 5°C to about 20°C, about 9°C to about 15°C, about 10°C to about 15°C, about 11°C to about 14°C, about 11°C to about 13°C, about 5°C, about 6°C, about 7°C, about 8°C, about 9°C, about 10°C, about 11°C, about 12°C, about 13°C, about 14°C, about 15°C, about 16°C, about 17°C, about 18°C, about 19°C, or about 20°C. In a specific example, the secondary drying temperature can be about 12°C. Alternatively, the holding temperature can be between about −10°C and about 30°C, between about −10°C and about 25°C, between about −10°C and about 20°C, between about −10°C and about 10°C, between about −5°C and about 30°C, between about −5°C and about 25°C, between about −5°C and about 20°C, between about −5°C and about 15°C, between about −5°C and about 10°C, between about −5°C and about 5°C, between about −4°C and about 4°C, between about −3°C and about 3°C, between about −2°C and about 2°C, between about −1°C and about 1°C, or about 0°C. In a specific example, the holding temperature can be between about −5°C and about 5°C or about 0°C.

[00186] The pressure (vacuum conditions) can be any suitable pressure. In some cases, the pressure is in the same range as for the primary drying step. However, some cycles during secondary drying may have full vacuum. For example, the pressure can be from about 0.140 to about 0.020, 0.140 to about 0.030, 0.140 to about 0.040, 0.140 to about 0.050, about 0.100 to about 0.060, about 0.100 to about 0.070, about 0.100 to about 0.080, about 0.099 to about 0.081, about 0.098 to about 0.082, about 0.097 to about 0.083, about 0.096 to about 0.084, about 0.095 to about 0.085, about 0.094 to about 0.086, about 0.093 to about 0.087, about 0.092 to about 0.088, about 0.091 to about 0.089, about 0.090 mbar, or about 0.120 mbar. In a specific example, the pressure is about 0.090 mbar.

[00187] In a specific example, the holding temperature in the secondary drying step is between about −5°C and about 5°C (e.g., about 0°C), the secondary drying step comprises increasing the temperature to the holding temperature at a rate of about 0.2°C per minute, and the secondary drying step secondary drying step comprises holding the composition at the holding temperature for about 5 hours to about 6 hours.

[00188] The secondary drying step can result in a lyophilized product having any desired residual moisture. For example, the residual moisture can be no more than about 7.0%, about 6.9%, about 6.8%, about 6.7%. about 6.6%, about 6.5%, about 6.4%, about 6.3%, about 6.2%, about 6.1%, about 6.0%, about 5.9%, about 5.8%, about 5.7%, about 5.6%, about 5.5%, about

5.4%, about 5.3%, about 5.2%, about 5.1%, about 5.0%, about 4.9%, about 4.8%, about 4.7%, about 4.6%, about 4.5%, about 4.4%, about 4.3%, about 4.2%, about 4.1%, about 4.0%, about 3.9%, about 3.8%, about 3.7%, about 3.6%, about 3.5%, about 3.4%, about 3.3%, about 3.2%, about 3.1%, about 3.0%, about 2.9%, about 2.8%, about 2.7%, about 2.6%, about 2.5%, about 2.4%, about 2.3%, about 2.2%, about 2.1%, about 2.0%, about 1.9%, about 1.8%, about 1.7%, about 1.6%, about 1.5%, about 1.4%, about 1.3%, about 1.2%, about 1.1%, or about 1.0%. Alternatively, the residual moisture can be at least about 7.0%, about 6.9%, about 6.8%, about 6.7%. about 6.6%, about 6.5%, about 6.4%, about 6.3%, about 6.2%, about 6.1%, about 6.0%, about 5.9%, about 5.8%, about 5.7%, about 5.6%, about 5.5%, about 5.4%, about 5.3%, about 5.2%, about 5.1%, about 5.0%, about 4.9%, about 4.8%, about 4.7%, about 4.6%, about 4.5%, about 4.4%, about 4.3%, about 4.2%, about 4.1%, about 4.0%, about 3.9%, about 3.8%, about 3.7%, about 3.6%, about 3.5%, about 3.4%, about 3.3%, about 3.2%, about 3.1%, about 3.0%, about 2.9%, about 2.8%, about 2.7%, about 2.6%, about 2.5%, about 2.4%, about 2.3%, about 2.2%, about 2.1%, about 2.0%, about 1.9%, about 1.8%, about 1.7%, about 1.6%, about 1.5%, about 1.4%, about 1.3%, about 1.2%, about 1.1%, or about 1.0%. In a specific example, the residual moisture can be at least about 1%, at least about 1.5%, or at about least 2% and no more than about 7%. Alternatively, the residual moisture can be between about 1% to about 7%, about 1% to about 6.5%, about 1% to about 6%, about 1% to about 5.5%, about 1% to about 5%, about 1.5% to about 7%, about 1.5% to about 6.5%, about 1.5% to about 6%, about 1.5% to about 5.5%, about 1.5% to about 5%, about 1.5% to about 4.5%, about 2% to about 7%, about 2% to about 6.5%, about 2% to about 6%, about 2% to about 5.5%, about 2% to about 5%, about 2% to about 4.5%, about 2% to about 4%, about 2% to about 3%, or about 3% to about 4%. In a specific example, the residual moisture can be about 3% to about 4%, about 3.1% to about 3.9%, about 3.2% to about 3.8%, about 3.3% to about 3.7%, about 3.4% to about 3.6%, or about 3.5%. In a specific example, the residual moisture is at least about 2%, at least about 2.5%, or at least about 3%. In another specific example, the residual moisture is between about 1% and about 5%, between about 2% and about 4%, between about 2.5% and about 3.5%, between about 2.5% and about 4%, between about 3% and about 4%, or between about 3% and about 3.5%.

F. Storage and Reconstitution of Lyophilized Bacteria or *Listeria*

[00189] The resulting lyophilized bacteria or *Listeria* can be a lyophilized composition comprising any combination of the components listed in the formulation section. In one example, the lyophilized composition comprises a *Listeria* strain, a buffer (e.g., a phosphate), and an excipient (e.g., sucrose). Optionally, the lyophilized composition does not comprise one or more or all of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA). Optionally, the lyophilized composition does not comprise one or more or all of the optional components listed in the formulation section.

[00190] The resulting lyophilized bacteria or *Listeria* can be a lyophilized composition with any of the residual moisture levels listed elsewhere herein. As one example, the residual moisture level can be between about 1% and about 5%, between about 2% and about 4%, or between about 3% and about 4%.

[00191] The lyophilized bacteria can be stored under any suitable conditions, including any suitable temperature, relative humidity, and atmospheric oxygen level, which are well-known. The lyophilized bacteria or *Listeria* can exhibit viability upon reconstitution of at least about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% after storage for a defined amount of time. The reconstitution can follow storage of the lyophilized bacteria or *Listeria* for example, for 2 days, 3 days, 4 days, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 5 months, 6 months, 9 months, 12 months (1 year), 15 months, 18 months, 21 months, or 24 months (2 years).

[00192] The storage temperature of the lyophilized bacteria or *Listeria* can be for example, between about 0°C and about 10°C, about 1°C and about 9°C, about 2°C and about 8°C, about 2°C and about 6°C, or about 3°C and about 5°C. In a specific example, the storage temperature of can be between about 2°C and about 8°C, or the storage temperature can be about 4°C. In another example, the storage temperature can be between about −15°C and about −25°C, about −16°C and about −24°C, about −17°C and about −23°C, about −18°C and about −22°C, or about −19°C and about −21°C. In a specific example, the storage temperature of can be about −20°C.

[00193] For example, the lyophilized bacteria or *Listeria* can show at least about 60%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% viability after storage

at about 2-8°C (e.g., 4°C) or about −20°C for about 6 months, about 9 months, about 12 months, about 18 months, or about 24 months. The lyophilized bacteria or *Listeria* can show at least about 60%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% viability after storage at about 30°C, at about room temperature (i.e., about 20-25°C (e.g., 20°C, 21°C, 22°C, 23°C, 24°C, or 25°C)), at about 2-8°C (e.g., 4°C) or about −20°C for about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 9 months, about 12 months, about 18 months, or about 24 months. As one example, the lyophilized bacteria or *Listeria* can show at least about 75% to about 80% viability at 2-8°C after 6 months. As another example, the lyophilized bacteria or *Listeria* can show at least about 95% to about 100% viability at −20°C after 9 months. As another example, the lyophilized bacteria or *Listeria* can show at least about 80% to about 90% viability at room temperature or at 30°C after 2 months. As another example, the lyophilized bacteria or *Listeria* can show at least about 60%, 65%, 70%, 75%, 80%, 85%, or 90% viability at about −20°C after about 12 months, 18 months, or 24 months. As another example, the lyophilized bacteria or *Listeria* can show at least about 60%, 65%, 70%, 75%, 80% viability at about 2-8°C after about 12 months, 18 months, or 24 months. As another example, the lyophilized bacteria or *Listeria* can show at least about 60%, 65%, 70%, 75%, or 80% viability at about 2-8°C after about 12 months, 18 months, or 24 months.

[00194] After storage, the lyophilized bacteria or *Listeria* strain can optionally be reconstituted with a solvent or diluent (e.g., water). As one example, the solvent or diluent can be appropriate media for culturing the bacteria or *Listeria* strain. Methods for reconstitution and rehydration of lyophilized bacteria or *Listeria* strains are well-known. In one example, the volume of solvent used is the volume of pre-lyophilization solution used to make the lyophilized bacteria or *Listeria* strain. In another example, the volume of solvent used is more than the volume of pre-lyophilization solution used to make the lyophilized bacteria or *Listeria* strain. In another example, the volume of solvent used is less than the volume of pre-lyophilization solution used to make the lyophilized bacteria or *Listeria* strain.

[00195] The reconstitution time can be any suitable reconstitution time. For example, the reconstitution time can be less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, or 30 minutes. In a specific example, the reconstitution time is less than about 2 minutes.

III. Recombinant Bacteria or Listeria *Strains*

[00196] The lyophilized compositions disclosed herein and the compositions that undergo the lyophilization methods disclosed herein comprise bacteria strains, such as a *Listeria* strain. Such bacteria strains can be recombinant bacteria strains. Such recombinant bacteria strains can comprise a recombinant fusion polypeptide disclosed herein or a nucleic acid encoding the recombinant fusion polypeptide as disclosed elsewhere herein. In some embodiments, the bacteria strain is a *Listeria* strain, such as a *Listeria monocytogenes* (*Lm*) strain. *Lm* has a number of inherent advantages as a vaccine vector. The bacterium grows very efficiently *in vitro* without special requirements, and it lacks LPS, which is a major toxicity factor in gram-negative bacteria, such as *Salmonella*. Genetically attenuated *Lm* vectors also offer additional safety as they can be readily eliminated with antibiotics, in case of serious adverse effects, and unlike some viral vectors, no integration of genetic material into the host genome occurs.

[00197] The recombinant *Listeria* strain can be any *Listeria* strain. Examples of suitable *Listeria* strains include *Listeria seeligeri*, *Listeria grayi*, *Listeria ivanovii*, *Listeria murrayi*, *Listeria welshimeri*, *Listeria monocytogenes* (*Lm*), or any other known *Listeria* species. In some embodiments, the recombinant listeria strain is a strain of the species *Listeria monocytogenes*. Examples of *Listeria monocytogenes* strains include the following: *L. monocytogenes* 10403S wild type (*see, e.g.*, Bishop and Hinrichs (1987) *J Immunol* 139:2005-2009; Lauer et al. (2002) *J Bact* 184:4177-4186); *L. monocytogenes* DP-L4056, which is phage cured (*see, e.g.*, Lauer et al. (2002) *J Bact* 184:4177-4186); *L. monocytogenes* DP-L4027, which is phage cured and has an *hly* gene deletion (*see, e.g.*, Lauer et al. (2002) *J Bact* 184:4177- 4186; Jones and Portnoy (1994) *Infect Immunity* 65:5608-5613); *L. monocytogenes* DP-L4029, which is phage cured and has an *actA* gene deletion (*see, e.g.*, Lauer et al. (2002) *J Bact* 184:4177-4186; Skoble et al. (2000) *J Cell Biol* 150:527- 538); *L. monocytogenes* DP-L4042 (delta PEST) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci. USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP-L4097 (LLO-S44A) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP- L4364 (delta *lplA*;

lipoate protein ligase) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP-L4405 (delta *inlA*) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP-L4406 (delta *inlB*) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* CS-LOOOl (delta *actA*; delta *inlB*) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* CS-L0002 (delta *actA*; delta *lplA*) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* CS-L0003 (LLO L461T; delta *lplA*) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP-L4038 (delta *actA*; LLO L461T) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); *L. monocytogenes* DP-L4384 (LLO S44A; LLO L461T) (*see, e.g.*, Brockstedt et al. (2004) *Proc Natl Acad Sci USA* 101:13832-13837 and supporting information); a *L. monocytogenes* strain with an *lplA1* deletion (encoding lipoate protein ligase LplA1) (*see, e.g.*, O'Riordan et al. (2003) *Science* 302:462-464); *L. monocytogenes* DP-L4017 (10403S with LLO L461T) (*see, e.g.*, US 7,691,393); *L. monocytogenes* EGD (*see, e.g.*, GenBank Accession No. AL591824). In another embodiment, the Listeria strain is L. monocytogenes EGD-e (see GenBank Accession No. NC_003210; ATCC Accession No. BAA-679); *L. monocytogenes* DP-L4029 (*actA* deletion, optionally in combination with *uvrAB* deletion (DP-L4029uvrAB) (*see, e.g.*, US 7,691,393); *L. monocytogenes actA-/inlB* - double mutant (*see, e.g.*, ATCC Accession No. PTA-5562); *L. monocytogenes lplA* mutant or *hly* mutant (*see, e.g.*, US 2004/0013690); *L. monocytogenes dal/dat* double mutant (*see, e.g.*, US 2005/0048081). Other *L. monocytogenes* strains includes those that are modified (e.g., by a plasmid and/or by genomic integration) to contain a nucleic acid encoding one of, or any combination of, the following genes: *hly* (LLO; listeriolysin); *iap* (p60); *inlA*; *inlB*; *inlC*; *dal* (alanine racemase); *dat* (D-amino acid aminotransferase); *plcA*; *plcB*; *actA*; or any nucleic acid that mediates growth, spread, breakdown of a single walled vesicle, breakdown of a double walled vesicle, binding to a host cell, or uptake by a host cell. Each of the above references is herein incorporated by reference in its entirety for all purposes.

[00198] The recombinant bacteria or *Listeria* can have wild-type virulence, can have attenuated virulence, or can be avirulent. For example, a recombinant *Listeria* of can be

sufficiently virulent to escape the phagosome or phagolysosome and enter the cytosol. Such *Listeria* strains can also be live-attenuated *Listeria* strains, which comprise at least one attenuating mutation, deletion, or inactivation as disclosed elsewhere herein. In some embodiments, the recombinant *Listeria* is an attenuated auxotrophic strain. An auxotrophic strain is one that is unable to synthesize a particular organic compound required for its growth. Examples of such strains are described in US 8,114,414, herein incorporated by reference in its entirety for all purposes.

[00199] In some embodiments, the recombinant *Listeria* strain lacks antibiotic resistance genes. For example, such recombinant *Listeria* strains can comprise a plasmid that does not encode an antibiotic resistance gene. However, some recombinant *Listeria* strains provided herein comprise a plasmid comprising a nucleic acid encoding an antibiotic resistance gene. Antibiotic resistance genes may be used in the conventional selection and cloning processes commonly employed in molecular biology and vaccine preparation. Exemplary antibiotic resistance genes include gene products that confer resistance to ampicillin, penicillin, methicillin, streptomycin, erythromycin, kanamycin, tetracycline, chloramphenicol (CAT), neomycin, hygromycin, and gentamicin.

A. Bacteria or *Listeria* Strains Comprising Recombinant Fusion Polypeptides or Nucleic Acids Encoding Recombinant Fusion Polypeptides

[00200] The recombinant bacteria strains (e.g., *Listeria* strains) disclosed herein comprise a recombinant fusion polypeptide disclosed herein or a nucleic acid encoding the recombinant fusion polypeptide as disclosed elsewhere herein.

[00201] In bacteria or *Listeria* strains comprising a nucleic acid encoding a recombinant fusion protein, the nucleic acid can be codon optimized. Examples of optimal codons utilized by *L. monocytogenes* for each amino acid are shown US 2007/0207170, herein incorporated by reference in its entirety for all purposes. A nucleic acid is codon-optimized if at least one codon in the nucleic acid is replaced with a codon that is more frequently used by *L. monocytogenes* for that amino acid than the codon in the original sequence.

[00202] The nucleic acid can be present in an episomal plasmid within the bacteria or *Listeria* strain and/or the nucleic acid can be genomically integrated in the bacteria or *Listeria* strain. Some recombinant bacteria or *Listeria* strains comprise two separate nucleic acids encoding two

recombinant fusion polypeptides as disclosed herein: one nucleic acid in an episomal plasmid, and one genomically integrated in the bacteria or *Listeria* strain.

[00203] The episomal plasmid can be one that is stably maintained *in vitro* (in cell culture), *in vivo* (in a host), or both *in vitro* and *in vivo*. If in an episomal plasmid, the open reading frame encoding the recombinant fusion polypeptide can be operably linked to a promoter/regulatory sequence in the plasmid. If genomically integrated in the bacteria or *Listeria* strain, the open reading frame encoding the recombinant fusion polypeptide can be operably linked to an exogenous promoter/regulatory sequence or to an endogenous promoter/regulatory sequence. Examples of promoters/regulatory sequences useful for driving constitutive expression of a gene are well-known and include, for example, an *hly*, *hlyA*, *actA*, *prfA*, and *p60* promoters of *Listeria*, the *Streptococcus bac* promoter, the *Streptomyces griseus sgiA* promoter, and the *B. thuringiensis phaZ* promoter. In some cases, an inserted gene of interest is not interrupted or subjected to regulatory constraints which often occur from integration into genomic DNA, and in some cases, the presence of the inserted heterologous gene does not lead to rearrangement or interruption of the cell's own important regions.

[00204] Such recombinant bacteria or *Listeria* strains can be made by transforming a bacteria or *Listeria* strain or an attenuated bacteria or *Listeria* strain described elsewhere herein with a plasmid or vector comprising a nucleic acid encoding the recombinant fusion polypeptide. The plasmid can be an episomal plasmid that does not integrate into a host chromosome. Alternatively, the plasmid can be an integrative plasmid that integrates into a chromosome of the bacteria or *Listeria* strain. The plasmids used herein can also be multicopy plasmids. Methods for transforming bacteria are well-known, and include calcium-chloride competent cell-based methods, electroporation methods, bacteriophage-mediated transduction, chemical transformation techniques, and physical transformation techniques. *See, e.g.*, de Boer et al. (1989) *Cell* 56:641-649; Miller et al. (1995) *FASEB J.* 9:190-199; Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Ausubel et al. (1997) Current Protocols in Molecular Biology, John Wiley & Sons, New York; Gerhardt et al., eds., 1994, Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, D.C.; and Miller, 1992, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., each of which is herein incorporated by reference in its entirety for all purposes.

[00205] Bacteria or *Listeria* strains with genomically integrated heterologous nucleic acids can be made, for example, by using a site-specific integration vector, whereby the bacteria or *Listeria* comprising the integrated gene is created using homologous recombination. The integration vector can be any site-specific integration vector that is capable of infecting a bacteria or *Listeria* strain. Such an integration vector can comprise, for example, a PSA attPP' site, a gene encoding a PSA integrase, a U153 attPP' site, a gene encoding a U153 integrase, an A118 attPP' site, a gene encoding an A118 integrase, or any other known attPP' site or any other phage integrase.

[00206] Such bacteria or *Listeria* strains comprising an integrated gene can also be created using any other known method for integrating a heterologous nucleic acid into a bacteria or *Listeria* chromosome. Techniques for homologous recombination are well-known, and are described, for example, in Baloglu et al. (2005) *Vet Microbiol* 109(1-2):11-17); Jiang et al. 2005) *Acta Biochim Biophys Sin (Shanghai)* 37(1):19-24), and US 6,855,320, each of which is herein incorporated by reference in its entirety for all purposes.

[00207] Integration into a bacteria or *Listerial* chromosome can also be achieved using transposon insertion. Techniques for transposon insertion are well-known, and are described, for example, for the construction of DP-L967 by Sun et al. (1990) *Infection and Immunity* 58: 3770-3778, herein incorporated by reference in its entirety for all purposes. Transposon mutagenesis can achieve stable genomic insertion, but the position in the genome where the heterologous nucleic acids has been inserted is unknown.

[00208] Integration into a bacterial or *Listerial* chromosome can also be achieved using phage integration sites (*see, e.g.*, Lauer et al. (2002) *J Bacteriol* 184(15):4177-4186, herein incorporated by reference in its entirety for all purposes). For example, an integrase gene and attachment site of a bacteriophage (e.g., U153 or PSA listeriophage) can be used to insert a heterologous gene into the corresponding attachment site, which may be any appropriate site in the genome (e.g. *comK* or the 3′ end of the *arg* tRNA gene). Endogenous prophages can be cured from the utilized attachment site prior to integration of the heterologous nucleic acid. Such methods can result, for example, in single-copy integrants. In order to avoid a "phage curing step," a phage integration system based on PSA phage can be used (*see, e.g.*, Lauer et al. (2002) *J Bacteriol* 184:4177-4186, herein incorporated by reference in its entirety for all purposes). Maintaining the integrated gene can require, for example, continuous selection by antibiotics.

Alternatively, a phage-based chromosomal integration system can be established that does not require selection with antibiotics. Instead, an auxotrophic host strain can be complemented. For example, a phage-based chromosomal integration system for clinical applications can be used, where a host strain that is auxotrophic for essential enzymes, including, for example, D-alanine racemase is used (e.g., *Lm dal*(−)*dat*(−)).

[00209] Conjugation can also be used to introduce genetic material and/or plasmids into bacteria. Methods for conjugation are well-known, and are described, for example, in Nikodinovic et al. (2006) *Plasmid* 56(3):223-227 and Auchtung et al. (2005) *Proc Natl Acad Sci USA* 102(35):12554-12559, each of which is herein incorporated by reference in its entirety for all purposes.

[00210] In a specific example, a recombinant bacteria or *Listeria* strain can comprise a nucleic acid encoding a recombinant fusion polypeptide genomically integrated into the bacteria or *Listeria* genome as an open reading frame with an endogenous *actA* sequence (encoding an ActA protein) or an endogenous *hly* sequence (encoding an LLO protein). For example, the expression and secretion of the fusion polypeptide can be under the control of the endogenous *actA* promoter and ActA signal sequence or can be under the control of the endogenous *hly* promoter and LLO signal sequence. As another example, the nucleic acid encoding a recombinant fusion polypeptide can replace an *actA* sequence encoding an ActA protein or an *hly* sequence encoding an LLO protein.

[00211] Selection of recombinant bacteria or *Listeria* strains can be achieved by any means. For example, antibiotic selection can be used. Antibiotic resistance genes may be used in the conventional selection and cloning processes commonly employed in molecular biology and vaccine preparation. Exemplary antibiotic resistance genes include gene products that confer resistance to ampicillin, penicillin, methicillin, streptomycin, erythromycin, kanamycin, tetracycline, chloramphenicol (CAT), neomycin, hygromycin, and gentamicin. Alternatively, auxotrophic strains can be used, and an exogenous metabolic gene can be used for selection instead of or in addition to an antibiotic resistance gene. As an example, in order to select for auxotrophic bacteria comprising a plasmid encoding a metabolic enzyme or a complementing gene provided herein, transformed auxotrophic bacteria can be grown in a medium that will select for expression of the gene encoding the metabolic enzyme (e.g., amino acid metabolism

gene) or the complementing gene. Alternatively, a temperature-sensitive plasmid can be used to select recombinants or any other known means for selecting recombinants.

B. Attenuation of Bacteria or *Listeria* Strains

[00212] The recombinant bacteria strains (e.g., recombinant *Listeria* strains) disclosed herein can be attenuated. The term "attenuation" encompasses a diminution in the ability of the bacterium to cause disease in a host animal. For example, the pathogenic characteristics of an attenuated *Listeria* strain may be lessened compared with wild-type *Listeria*, although the attenuated *Listeria* is capable of growth and maintenance in culture. In some embodiments, using as an example the intravenous inoculation of BALB/c mice with an attenuated *Listeria*, the lethal dose at which 50% of inoculated animals survive (LD_{50}) is increased above the LD_{50} of wild-type *Listeria* by at least about 10-fold, by at least about 100-fold, by at least about 1,000 fold, by at least about 10,000 fold, or by at least about 100,000-fold. An attenuated strain of *Listeria* is thus one that does not kill an animal to which it is administered, or is one that kills the animal only when the number of bacteria administered is vastly greater than the number of wild-type non-attenuated bacteria which would be required to kill the same animal. An attenuated bacterium should also be construed to mean one which is incapable of replication in the general environment because the nutrient required for its growth is not present therein. Thus, the bacterium is limited to replication in a controlled environment wherein the required nutrient is provided. Attenuated strains are environmentally safe in that they are incapable of uncontrolled replication

(1) Methods of Attenuating Bacteria and Listeria *Strains*

[00213] Attenuation can be accomplished by any known means. For example, such attenuated strains can be deficient in one or more endogenous virulence genes or one or more endogenous metabolic genes. Examples of such genes are disclosed herein, and attenuation can be achieved by inactivation of any one of or any combination of the genes disclosed herein. Inactivation can be achieved, for example, through deletion or through mutation (e.g., an inactivating mutation). The term "mutation" includes any type of mutation or modification to the sequence (nucleic acid or amino acid sequence) and may encompass a deletion, a truncation, an insertion, a substitution, a disruption, or a translocation. For example, a mutation can include a frameshift mutation, a

mutation which causes premature termination of a protein, or a mutation of regulatory sequences which affect gene expression. Mutagenesis can be accomplished using recombinant DNA techniques or using traditional mutagenesis technology using mutagenic chemicals or radiation and subsequent selection of mutants. In some embodiments, deletion mutants are used because of the accompanying low probability of reversion. The term "metabolic gene" refers to a gene encoding an enzyme involved in or required for synthesis of a nutrient utilized or required by a host bacteria. For example, the enzyme can be involved in or required for the synthesis of a nutrient required for sustained growth of the host bacteria. The term "virulence" gene includes a gene whose presence or activity in an organism's genome that contributes to the pathogenicity of the organism (e.g., enabling the organism to achieve colonization of a niche in the host (including attachment to cells), immunoevasion (evasion of host's immune response), immunosuppression (inhibition of host's immune response), entry into and exit out of cells, or obtaining nutrition from the host).

[00214] A specific example of such an attenuated strain is *Listeria monocytogenes* (*Lm*) *dal*(−)*dat*(−) (*Lmdd*). Another example of such an attenuated strain is *Lm dal*(−)*dat*(−)Δ*actA* (*LmddA*). *See, e.g.,* US 2011/0142791, herein incorporated by references in its entirety for all purposes. *LmddA* is based on a *Listeria* strain which is attenuated due to the deletion of the endogenous virulence gene *actA*. Such strains can retain a plasmid for antigen expression *in vivo* and *in vitro* by complementation of the *dal* gene. Alternatively, the *LmddA* can be a *dal/dat/actA Listeria* having mutations in the endogenous *dal, dat*, and *actA* genes. Such mutations can be, for example, a deletion or other inactivating mutation.

[00215] Another specific example of an attenuated strain is *Lm prfA*(−) or a strain having a partial deletion or inactivating mutation in the *prfA* gene. The PrfA protein controls the expression of a regulon comprising essential virulence genes required by *Lm* to colonize its vertebrate hosts; hence the prfA mutation strongly impairs PrfA ability to activate expression of PrfA-dependent virulence genes.

[00216] Yet another specific example of an attenuated strain is *Lm inlB*(−)*actA*(−) in which two genes critical to the bacterium's natural virulence—*internalin B* and *act A*—are deleted.

[00217] Other examples of attenuated bacteria or *Listeria* strains include bacteria or *Listeria* strains deficient in one or more endogenous virulence genes. Examples of such genes include *actA*, *prfA*, *plcB*, *plcA*, *inlA*, *inlB*, *inlC*, *inlJ*, and *bsh* in *Listeria*. Attenuated *Listeria* strains can

also be the double mutant or triple mutant of any of the above-mentioned strains. Attenuated *Listeria* strains can comprise a mutation or deletion of each one of the genes, or comprise a mutation or deletion of, for example, up to ten of any of the genes provided herein (e.g., including the *actA*, *prfA*, and *dal/dat* genes). For example, an attenuated *Listeria* strain can comprise a mutation or deletion of an endogenous *internalin C* (*inlC*) gene and/or a mutation or deletion of an endogenous *actA* gene. Alternatively, an attenuated *Listeria* strain can comprise a mutation or deletion of an endogenous *internalin B* (*inlB*) gene and/or a mutation or deletion of an endogenous *actA* gene. Alternatively, an attenuated *Listeria* strain can comprise a mutation or deletion of endogenous *inlB*, *inlC*, and *actA* genes. Translocation of *Listeria* to adjacent cells is inhibited by the deletion of the endogenous *actA* gene and/or the endogenous *inlC* gene or endogenous *inlB* gene, which are involved in the process, thereby resulting in high levels of attenuation with increased immunogenicity and utility as a strain backbone. An attenuated *Listeria* strain can also be a double mutant comprising mutations or deletions of both *plcA* and *plcB*. In some cases, the strain can be constructed from the EGD *Listeria* backbone.

[00218] A bacteria or *Listeria* strain can also be an auxotrophic strain having a mutation in a metabolic gene. As one example, the strain can be deficient in one or more endogenous amino acid metabolism genes. For example, the generation of auxotrophic strains of *Listeria* deficient in D-alanine, for example, may be accomplished in a number of ways that are well-known, including deletion mutations, insertion mutations, frameshift mutations, mutations which cause premature termination of a protein, or mutation of regulatory sequences which affect gene expression. In some embodiments, deletion mutants are used because of the accompanying low probability of reversion of the auxotrophic phenotype. As an example, mutants of D-alanine which are generated according to the protocols presented herein may be tested for the ability to grow in the absence of D-alanine in a simple laboratory culture assay. Those mutants which are unable to grow in the absence of this compound can be selected.

[00219] Examples of endogenous amino acid metabolism genes include a vitamin synthesis gene, a gene encoding pantothenic acid synthase, a D-glutamic acid synthase gene, a D-alanine amino transferase (*dat*) gene, a D-alanine racemase (*dal*) gene, *dga*, a gene involved in the synthesis of diaminopimelic acid (DAP), a gene involved in the synthesis of Cysteine synthase A (*cysK*), a vitamin-B12 independent methionine synthase, *trpA*, *trpB*, *trpE*, *asnB*, *gltD*, *gltB*, *leuA*, *argG*, and *thrC*. The *Listeria* strain can be deficient in two or more such genes (e.g., *dat* and

dal). D-glutamic acid synthesis is controlled in part by the *dal* gene, which is involved in the conversion of D-glu + pyr to alpha-ketoglutarate + D-ala, and the reverse reaction.

[00220] As another example, an attenuated *Listeria* strain can be deficient in an endogenous synthase gene, such as an amino acid synthesis gene. Examples of such genes include *folP*, a gene encoding a dihydrouridine synthase family protein, *ispD*, *ispF*, a gene encoding a phosphoenolpyruvate synthase, *hisF*, *hisH*, *fliI*, a gene encoding a ribosomal large subunit pseudouridine synthase, *ispD*, a gene encoding a bifunctional GMP synthase/glutamine amidotransferase protein, *cobS*, *cobB*, *cbiD*, a gene encoding a uroporphyrin-III C-methyltransferase/uroporphyrinogen-III synthase, *cobQ*, *uppS*, *truB*, *dxs*, *mvaS*, *dapA*, *ispG*, *folC*, a gene encoding a citrate synthase, *argJ*, a gene encoding a 3-deoxy-7-phosphoheptulonate synthase, a gene encoding an indole-3-glycerol-phosphate synthase, a gene encoding an anthranilate synthase/glutamine amidotransferase component, *menB*, a gene encoding a menaquinone-specific isochorismate synthase, a gene encoding a phosphoribosylformylglycinamidine synthase I or II, a gene encoding a phosphoribosylaminoimidazole-succinocarboxamide synthase, *carB*, *carA*, *thyA*, *mgsA*, *aroB*, *hepB*, *rluB*, *ilvB*, *ilvN*, *alsS*, *fabF*, *fabH*, a gene encoding a pseudouridine synthase, *pyrG*, *truA*, *pabB*, and an ATP synthase gene (e.g., *atpC, atpD-2, aptG, atpA-2*, and so forth).

[00221] Attenuated *Listeria* strains can be deficient in endogenous *phoP*, *aroA*, *aroC*, *aroD*, or *plcB*. As yet another example, an attenuated *Listeria* strain can be deficient in an endogenous peptide transporter. Examples include genes encoding an ABC transporter/ATP-binding/permease protein, an oligopeptide ABC transporter/oligopeptide-binding protein, an oligopeptide ABC transporter/permease protein, a zinc ABC transporter/zinc-binding protein, a sugar ABC transporter, a phosphate transporter, a ZIP zinc transporter, a drug resistance transporter of the *EmrB/QacA* family, a sulfate transporter, a proton-dependent oligopeptide transporter, a magnesium transporter, a formate/nitrite transporter, a spermidine/putrescine ABC transporter, a Na/Pi-cotransporter, a sugar phosphate transporter, a glutamine ABC transporter, a major facilitator family transporter, a glycine betaine/L-proline ABC transporter, a molybdenum ABC transporter, a techoic acid ABC transporter, a cobalt ABC transporter, an ammonium transporter, an amino acid ABC transporter, a cell division ABC transporter, a manganese ABC transporter, an iron compound ABC transporter, a maltose/maltodextrin ABC transporter, a drug resistance transporter of the *Bcr/CflA* family, and a subunit of one of the above proteins.

[00222] Other attenuated bacteria and *Listeria* strains can be deficient in an endogenous metabolic enzyme that metabolizes an amino acid that is used for a bacterial growth process, a replication process, cell wall synthesis, protein synthesis, metabolism of a fatty acid, or for any other growth or replication process. Likewise, an attenuated strain can be deficient in an endogenous metabolic enzyme that can catalyze the formation of an amino acid used in cell wall synthesis, can catalyze the synthesis of an amino acid used in cell wall synthesis, or can be involved in synthesis of an amino acid used in cell wall synthesis. Alternatively, the amino acid can be used in cell wall biogenesis. Alternatively, the metabolic enzyme is a synthetic enzyme for D-glutamic acid, a cell wall component.

[00223] Other attenuated *Listeria* strains can be deficient in metabolic enzymes encoded by a D-glutamic acid synthesis gene, *dga*, an *alr* (alanine racemase) gene, or any other enzymes that are involved in alanine synthesis. Yet other examples of metabolic enzymes for which the *Listeria* strain can be deficient include enzymes encoded by *serC* (a phosphoserine aminotransferase), *asd* (aspartate betasemialdehyde dehydrogenase; involved in synthesis of the cell wall constituent diaminopimelic acid), the gene encoding gsaB- glutamate-1-semialdehyde aminotransferase (catalyzes the formation of 5-aminolevulinate from (S)-4-amino-5-oxopentanoate), *hemL* (catalyzes the formation of 5-aminolevulinate from (S)-4-amino-5-oxopentanoate), *aspB* (an aspartate aminotransferase that catalyzes the formation of oxalozcetate and L-glutamate from L-aspartate and 2-oxoglutarate), *argF-1* (involved in arginine biosynthesis), *aroE* (involved in amino acid biosynthesis), *aroB* (involved in 3-dehydroquinate biosynthesis), *aroD* (involved in amino acid biosynthesis), *aroC* (involved in amino acid biosynthesis), *hisB* (involved in histidine biosynthesis), *hisD* (involved in histidine biosynthesis), hisG (involved in histidine biosynthesis), *metX* (involved in methionine biosynthesis), *proB* (involved in proline biosynthesis), *argR* (involved in arginine biosynthesis), *argJ* (involved in arginine biosynthesis), *thil* (involved in thiamine biosynthesis), *LMOf2365_1652* (involved in tryptophan biosynthesis), *aroA* (involved in tryptophan biosynthesis), ilvD (involved in valine and isoleucine biosynthesis), *ilvC* (involved in valine and isoleucine biosynthesis), leuA (involved in leucine biosynthesis), *dapF* (involved in lysine biosynthesis), and *thrB* (involved in threonine biosynthesis) (all GenBank Accession No. NC_002973).

[00224] An attenuated *Listeria* strain can be generated by mutation of other metabolic enzymes, such as a tRNA synthetase. For example, the metabolic enzyme can be encoded by the

trpS gene, encoding tryptophanyl-tRNA synthetase. For example, the host strain bacteria can be Δ(*trpS aroA*), and both markers can be contained in an integration vector.

[00225] Other examples of metabolic enzymes that can be mutated to generate an attenuated *Listeria* strain include an enzyme encoded by *murE* (involved in synthesis of diaminopimelic acid; GenBank Accession No: NC_003485), LMOf2365_2494 (involved in teichoic acid biosynthesis), *WecE* (Lipopolysaccharide biosynthesis protein rffA; GenBank Accession No: AE014075.1), or *amiA* (an N-acetylmuramoyl-L-alanine amidase). Yet other examples of metabolic enzymes include aspartate aminotransferase, histidinol-phosphate aminotransferase (GenBank Accession No. NP_466347), or the cell wall teichoic acid glycosylation protein GtcA.

[00226] Other examples of metabolic enzymes that can be mutated to generate an attenuated *Listeria* strain include a synthetic enzyme for a peptidoglycan component or precursor. The component can be, for example, UDP-N-acetylmuramylpentapeptide, UDP-N-acetylglucosamine, MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol, GlcNAc-p-(1,4)-MurNAc-(pentapeptide)-pyrophosphorylundecaprenol, or any other peptidoglycan component or precursor.

[00227] Yet other examples of metabolic enzymes that can be mutated to generate an attenuated *Listeria* strain include metabolic enzymes encoded by *murG*, *murD*, *murA-1*, or *murA-2* (all set forth in GenBank Accession No. NC_002973). Alternatively, the metabolic enzyme can be any other synthetic enzyme for a peptidoglycan component or precursor. The metabolic enzyme can also be a trans-glycosylase, a trans-peptidase, a carboxy-peptidase, any other class of metabolic enzyme, or any other metabolic enzyme. For example, the metabolic enzyme can be any other *Listeria* metabolic enzyme or any other *Listeria monocytogenes* metabolic enzyme.

[00228] Other bacteria strains can be attenuated as described above for *Listeria* by mutating the corresponding orthologous genes in the other bacteria strains.

(2) Methods of Complementing Attenuated Bacteria and Listeria *Strains*

[00229] The attenuated bacteria or *Listeria* strains disclosed herein can further comprise a nucleic acid comprising a complementing gene or encoding a metabolic enzyme that complements an attenuating mutation (e.g., complements the auxotrophy of the auxotrophic *Listeria* strain). For example, a nucleic acid having a first open reading frame encoding a fusion

polypeptide as disclosed herein can further comprise a second open reading frame comprising the complementing gene or encoding the complementing metabolic enzyme. Alternatively, a first nucleic acid can encode the fusion polypeptide and a separate second nucleic acid can comprise the complementing gene or encode the complementing metabolic enzyme.

[00230] The complementing gene can be extrachromosomal or can be integrated into the bacteria or *Listeria* genome. For example, the auxotrophic *Listeria* strain can comprise an episomal plasmid comprising a nucleic acid encoding a metabolic enzyme. Such plasmids will be contained in the *Listeria* in an episomal or extrachromosomal fashion. Alternatively, the auxotrophic *Listeria* strain can comprise an integrative plasmid (i.e., integration vector) comprising a nucleic acid encoding a metabolic enzyme. Such integrative plasmids can be used for integration into a *Listeria* chromosome. In some embodiments, the episomal plasmid or the integrative plasmid lacks an antibiotic resistance marker.

[00231] The metabolic gene can be used for selection instead of or in addition to an antibiotic resistance gene. As an example, in order to select for auxotrophic bacteria comprising a plasmid encoding a metabolic enzyme or a complementing gene provided herein, transformed auxotrophic bacteria can be grown in a medium that will select for expression of the gene encoding the metabolic enzyme (e.g., amino acid metabolism gene) or the complementing gene. For example, a bacteria auxotrophic for D-glutamic acid synthesis can be transformed with a plasmid comprising a gene for D-glutamic acid synthesis, and the auxotrophic bacteria will grow in the absence of D-glutamic acid, whereas auxotrophic bacteria that have not been transformed with the plasmid, or are not expressing the plasmid encoding a protein for D-glutamic acid synthesis, will not grow. Similarly, a bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing a plasmid comprising a nucleic acid encoding an amino acid metabolism enzyme for D-alanine synthesis. Such methods for making appropriate media comprising or lacking necessary growth factors, supplements, amino acids, vitamins, antibiotics, and the like are well-known and are available commercially.

[00232] Once the auxotrophic bacteria comprising the plasmid encoding a metabolic enzyme or a complementing gene provided herein have been selected in appropriate medium, the bacteria can be propagated in the presence of a selective pressure. Such propagation can comprise growing the bacteria in media without the auxotrophic factor. The presence of the plasmid expressing the metabolic enzyme or the complementing gene in the auxotrophic bacteria ensures

that the plasmid will replicate along with the bacteria, thus continually selecting for bacteria harboring the plasmid. Production of the bacteria or *Listeria* strain can be readily scaled up by adjusting the volume of the medium in which the auxotrophic bacteria comprising the plasmid are growing.

[00233] In one specific example, the attenuated strain is a strain having a deletion of or an inactivating mutation in *dal* and *dat* (e.g., *Listeria monocytogenes* (*Lm*) *dal*(−)*dat*(−) (*Lmdd*) or *Lm dal*(−)*dat*(−)Δ*actA* (*LmddA*)), and the complementing gene encodes an alanine racemase enzyme (e.g., encoded by *dal* gene) or a D-amino acid aminotransferase enzyme (e.g., encoded by *dat* gene). An exemplary alanine racemase protein can have the sequence set forth in SEQ ID NO: 76 (encoded by SEQ ID NO: 78; GenBank Accession No: AF038438) or can be a homologue, variant, isoform, analog, fragment, fragment of a homologue, fragment of a variant, fragment of an analog, or fragment of an isoform of SEQ ID NO: 76 . The alanine racemase protein can also be any other *Listeria* alanine racemase protein. Alternatively, the alanine racemase protein can be any other gram-positive alanine racemase protein or any other alanine racemase protein. An exemplary D-amino acid aminotransferase protein can have the sequence set forth in SEQ ID NO: 77 (encoded by SEQ ID NO: 79; GenBank Accession No: AF038439) or can be a homologue, variant, isoform, analog, fragment, fragment of a homologue, fragment of a variant, fragment of an analog, or fragment of an isoform of SEQ ID NO: 77. The D-amino acid aminotransferase protein can also be any other *Listeria* D-amino acid aminotransferase protein. Alternatively, the D-amino acid aminotransferase protein can be any other gram-positive D-amino acid aminotransferase protein or any other D-amino acid aminotransferase protein.

[00234] In another specific example, the attenuated strain is a strain having a deletion of or an inactivating mutation in *prfA* (e.g., *Lm prfA*(−)), and the complementing gene encodes a PrfA protein. For example, the complementing gene can encode a mutant PrfA (D133V) protein that restores partial PrfA function. An example of a wild type PrfA protein is set forth in SEQ ID NO: 80 (encoded by nucleic acid set forth in SEQ ID NO: 81), and an example of a D133V mutant PrfA protein is set forth in SEQ ID NO: 82 (encoded by nucleic acid set forth in SEQ ID NO: 83). The complementing PrfA protein can be a homologue, variant, isoform, analog, fragment, fragment of a homologue, fragment of a variant, fragment of an analog, or fragment of an isoform of SEQ ID NO: 80 or 82. The PrfA protein can also be any other *Listeria* PrfA

protein. Alternatively, the PrfA protein can be any other gram-positive PrfA protein or any other PrfA protein.

[00235] In another example, the bacteria strain or *Listeria* strain can comprise a deletion of or an inactivating mutation in an *actA* gene, and the complementing gene can comprise an *actA* gene to complement the mutation and restore function to the *Listeria* strain.

[00236] Other auxotroph strains and complementation systems can also be adopted for the use with the methods and compositions provided herein.

IV. Recombinant Fusion Polypeptides

[00237] The recombinant fusion polypeptides in the recombinant bacteria or *Listeria* strains disclosed herein can be in any form. Some such fusion polypeptides can comprise a PEST-containing peptide fused to one or more disease-associated antigenic peptides. Other such recombinant fusion polypeptides can comprise one or more disease-associated antigenic peptides, and wherein the fusion polypeptide does not comprise a PEST-containing peptide.

[00238] Another example of a recombinant fusion polypeptides comprises from N-terminal end to C-terminal end a bacterial secretion sequence, a ubiquitin (Ub) protein, and one or more disease-associated antigenic peptides (i.e., in tandem, such as Ub-peptide1-peptide2). Alternatively, if two or more disease-associated antigenic peptides are used, a combination of separate fusion polypeptides can be used in which each antigenic peptide is fused to its own secretion sequence and Ub protein (e.g., Ub1-peptide1; Ub2-peptide2).

[00239] Nucleic acids (termed minigene constructs) encoding such recombinant fusion polypeptides are also disclosed. Such minigene nucleic acid constructs can further comprise two or more open reading frames linked by a Shine-Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. For example, a minigene nucleic acid construct can further comprise two to four open reading frames linked by a Shine-Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. Each open reading frame can encode a different polypeptide. In some nucleic acid constructs, the codon encoding the carboxy terminus of the fusion polypeptide is followed by two stop codons to ensure termination of protein synthesis.

[00240] The bacterial signal sequence can be a *Listerial* signal sequence, such as an Hly or an ActA signal sequence, or any other known signal sequence. In other cases, the signal sequence

can be an LLO signal sequence. An exemplary LLO signal sequence is set forth in SEQ ID NO: 97. The signal sequence can be bacterial, can be native to a host bacterium (e.g., *Listeria monocytogenes*, such as a secA1 signal peptide), or can be foreign to a host bacterium. Specific examples of signal peptides include an Usp45 signal peptide from *Lactococcus lactis*, a Protective Antigen signal peptide from *Bacillus anthracis*, a secA2 signal peptide such the p60 signal peptide from *Listeria monocytogenes*, and a Tat signal peptide such as a *B. subtilis* Tat signal peptide (e.g., PhoD). In specific examples, the secretion signal sequence is from a *Listeria* protein, such as an $ActA_{300}$ secretion signal or an $ActA_{100}$ secretion signal. An exemplary ActA signal sequence is set forth in SEQ ID NO: 98.

[00241] The ubiquitin can be, for example, a full-length protein. The ubiquitin expressed from the nucleic acid construct provided herein can be cleaved at the carboxy terminus from the rest of the recombinant fusion polypeptide expressed from the nucleic acid construct through the action of hydrolases upon entry to the host cell cytosol. This liberates the amino terminus of the fusion polypeptide, producing a peptide in the host cell cytosol.

[00242] Selection of, variations of, and arrangement of antigenic peptides within a fusion polypeptide are discussed in detail elsewhere herein, and examples of disease-associated antigenic peptides are discussed in more detail elsewhere herein.

[00243] The recombinant fusion polypeptides can comprise one or more tags. For example, the recombinant fusion polypeptides can comprise one or more peptide tags N-terminal and/or C-terminal to one or more antigenic peptides. A tag can be fused directly to an antigenic peptide or linked to an antigenic peptide via a linker (examples of which are disclosed elsewhere herein). Examples of tags include the following: FLAG tag; 2xFLAG tag; 3xFLAG tag; His tag, 6xHis tag; and SIINFEKL tag. An exemplary SIINFEKL tag is set forth in SEQ ID NO: 16 (encoded by any one of the nucleic acids set forth in SEQ ID NOS: 1-15). An exemplary 3×FLAG tag is set forth in SEQ ID NO: 32 (encoded by any one of the nucleic acids set forth in SEQ ID NOS: 17-31). An exemplary variant 3×FLAG tag is set forth in SEQ ID NO: 99. Two or more tags can be used together, such as a 2×FLAG tag and a SIINFEKL tag, a 3×FLAG tag and a SIINFEKL tag, or a 6×His tag and a SIINFEKL tag. If two or more tags are used, they can be located anywhere within the recombinant fusion polypeptide and in any order. For example, the two tags can be at the C-terminus of the recombinant fusion polypeptide, the two tags can be at the N-terminus of the recombinant fusion polypeptide, the two tags can be located internally

within the recombinant fusion polypeptide, one tag can be at the C-terminus and one tag at the N-terminus of the recombinant fusion polypeptide, one tag can be at the C-terminus and one internally within the recombinant fusion polypeptide, or one tag can be at the N-terminus and one internally within the recombinant fusion polypeptide. Other tags include chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), thioredoxin (TRX), and poly(NANP). Particular recombinant fusion polypeptides comprise a C-terminal SIINFEKL tag. Such tags can allow for easy detection of the recombinant fusion protein, confirmation of secretion of the recombinant fusion protein, or for following the immunogenicity of the secreted fusion polypeptide by following immune responses to these "tag" sequence peptides. Such immune response can be monitored using a number of reagents including, for example, monoclonal antibodies and DNA or RNA probes specific for these tags.

[00244] The recombinant fusion polypeptides disclosed herein can be expressed by recombinant *Listeria* strains or can be expressed and isolated from other vectors and cell systems used for protein expression and isolation. Recombinant *Listeria* strains comprising expressing such antigenic peptides can be used, for example in immunogenic compositions comprising such recombinant *Listeria* and in vaccines comprising the recombinant *Listeria* strain and an adjuvant. Expression of one or more antigenic peptides as a fusion polypeptides with a nonhemolytic truncated form of LLO, ActA, or a PEST-like sequence in host cell systems in *Listeria* strains and host cell systems other than *Listeria* can result in enhanced immunogenicity of the antigenic peptides.

[00245] Nucleic acids encoding such recombinant fusion polypeptides are also disclosed. The nucleic acid can be in any form. The nucleic acid can comprise or consist of DNA or RNA, and can be single-stranded or double-stranded. The nucleic acid can be in the form of a plasmid, such as an episomal plasmid, a multicopy episomal plasmid, or an integrative plasmid. Alternatively, the nucleic acid can be in the form of a viral vector, a phage vector, or in a bacterial artificial chromosome. Such nucleic acids can have one open reading frame or can have two or more open reading frames (e.g., an open reading frame encoding the recombinant fusion polypeptide and a second open reading frame encoding a metabolic enzyme). In one example, such nucleic acids can comprise two or more open reading frames linked by a Shine-Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. For example, a nucleic acid can comprise two to four open reading frames linked by a Shine-

Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. Each open reading frame can encode a different polypeptide. In some nucleic acids, the codon encoding the carboxy terminus of the fusion polypeptide is followed by two stop codons to ensure termination of protein synthesis.

A. Antigenic Peptides

[00246] Disease-associated peptides include peptides from proteins that are expressed in a particular disease. For example, such peptides may be from proteins that are expressed in a disease tissue but not in a corresponding normal tissue, or that are expressed at abnormally high levels in a disease tissue. The term "disease" as used herein is intended to be generally synonymous, and is used interchangeably with, the terms "disorder" and "condition" (as in medical condition), in that all reflect an abnormal condition of the human or animal body or of one of its parts that impairs normal functioning, is typically manifested by distinguishing signs and symptoms, and causes the human or animal to have a reduced duration or quality of life. Examples of disease-associated antigenic peptides can include Human Papilloma Virus (HPV) E7 or E6, a Prostate Specific Antigen (PSA), a chimeric Her2 antigen, Her2/neu chimeric antigen. Another example of a disease-associated antigenic peptide is a WT1 antigenic peptide. The Human Papilloma Virus can be HPV 16 or HPV 18. The antigenic peptide can also include HPV16 E6, HPV16 E7, HPV18 E6, HPV18 E7 antigens operably linked in tandem or HPV16 antigenic peptide operably linked in tandem to an HPV antigenic peptide.

[00247] The fusion polypeptide can include a single antigenic peptide or can includes two or more antigenic peptides. Each antigenic peptide can be of any length sufficient to induce an immune response, and each antigenic peptide can be the same length or the antigenic peptides can have different lengths. For example, an antigenic peptide disclosed herein can be 5-100, 15-50, or 21-27 amino acids in length, or 15-100, 15-95, 15-90, 15-85, 15-80, 15-75, 15-70, 15-65, 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, 20-100, 20-95, 20-90, 20-85, 20-80, 20-75, 20-70, 20-65, 20-60, 20-55, 20-50, 20-45, 20-40, 20-35, 20-30, 11-21, 15-21, 21-31, 31-41, 41-51, 51-61, 61-71, 71-81, 81-91, 91-101, 101-121, 121-141, 141-161, 161-181, 181-201, 8-27, 10-30, 10-40, 15-30, 15-40, 15-25, 1-10, 10-20, 20-30, 30-40, 1-100, 5-75, 5-50, 5-40, 5-30, 5-20, 5-15, 5-10, 1-75, 1-50, 1-40, 1-30, 1-20, 1-15, 1-10, 8-11, or 11-16 amino acids in length. For example, an antigenic peptide can be at least 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, or 60 amino acids in length. Some specific examples of antigenic peptides are 21 or 27 amino acids in length. Other antigenic peptides can be full-length proteins or fragments thereof.

[00248] As one example, an antigenic peptide can comprise a neoepitope. These neoepitopes can be, for example, patient-specific (i.e., subject-specific) cancer mutations. Antigenic peptides comprising neoepitopes can be generated in a process for creating a personalized immunotherapy comprising comparing nucleic acids extracted from a cancer sample from a subject to nucleic acids extracted from a normal or healthy reference sample in order to identify somatic mutations or sequence differences present in the cancer sample compared with the normal or healthy sample. For examples, these mutations or sequence differences can be somatic, nonsynonymous missense mutations, or somatic frameshift mutations, and can encode an expressed amino acid sequence. A peptide expressing such somatic mutations or sequence differences can be referred to as a "neoepitope." A cancer-specific neoepitope may refer to an epitope that is not present in a reference sample (such as a normal non-cancerous or germline cell or tissue) but is found in a cancer sample. This includes, for example, situations in which in a normal non-cancerous or germline cell a corresponding epitope is found, but due to one or more mutations in a cancer cell, the sequence of the epitope is changed so as to result in the neoepitope. A neoepitope can comprise a mutated epitope, and can comprise non-mutated sequence on either or both sides of the mutation.

[00249] As another example, antigenic peptides can comprise recurrent cancer mutations. Each antigenic peptide can comprise a single recurrent cancer mutation or can comprise two or more recurrent cancer mutations (e.g., two recurrent cancer mutations). For example, an antigenic peptide can comprise more than one recurrent cancer mutation (e.g., 2 or 3 recurrent cancer mutations) because of the close proximity of the mutated residues to each other in the cancer-associated protein. The recurrent cancer mutations can be any type of mutation (e.g., somatic missense mutation or frameshift mutation). For example, a recombinant fusion polypeptide disclosed herein can comprise a PEST-containing peptide fused to two or more antigenic peptides (i.e., in tandem, such as PEST-peptide1-peptide2) or can comprise two or more antigenic peptides not fused to a PEST-containing peptide, wherein each antigenic peptide comprises a single, recurrent cancer mutation (i.e., a single, recurrent change in the amino acid

sequence of a protein, or a sequence encoded by a single, different, nonsynonymous, recurrent cancer mutation in a gene), and wherein at least two of the antigenic peptides comprise different recurrent cancer mutations and are fragments of the same cancer-associated protein. Alternatively, each of the antigenic peptides can comprise a different recurrent cancer mutation from a different cancer-associated protein. Alternatively, a combination of separate fusion polypeptides can be used in which each antigenic peptide is fused (or is not fused) to its own PEST-containing peptide (e.g., PEST1-peptide1; PEST2-peptide2). Optionally, some or all of the fragments are non-contiguous fragments of the same cancer-associated protein. Non-contiguous fragments are fragments that do not occur sequentially in a protein sequence (e.g., the first fragment consists of residues 10-30, and the second fragment consists of residues 100-120; or the first fragment consists of residues 10-30, and the second fragment consists of residues 20-40). Optionally, each of the antigenic peptides comprises a different recurrent cancer mutation from a single type of cancer.

[00250] Recurrent cancer mutations can be from cancer-associated proteins. The term "cancer-associated protein" includes proteins having mutations that occur in multiple types of cancer, that occur in multiple subjects having a particular type of cancer, or that are correlated with the occurrence or progression of one or more types of cancer. For example, a cancer-associated protein can be an oncogenic protein (i.e., a protein with activity that can contribute to cancer progression, such as proteins that regulate cell growth), or it can be a tumor-suppressor protein (i.e., a protein that typically acts to alleviate the potential for cancer formation, such as through negative regulation of the cell cycle or by promoting apoptosis). In some embodiments, a cancer-associated protein has a "mutational hotspot." A mutational hotspot is an amino acid position in a protein-coding gene that is mutated (such as by somatic substitutions rather than other somatic abnormalities, such as translocations, amplifications, and deletions) more frequently than would be expected in the absence of selection. Such hotspot mutations can occur across multiple types of cancer and/or can be shared among multiple cancer patients. Mutational hotspots indicate selective pressure across a population of tumor samples. Tumor genomes contain recurrent cancer mutations that "drive" tumorigenesis by affecting genes (i.e., tumor driver genes) that confer selective growth advantages to the tumor cells upon alteration. Such tumor driver genes can be identified, for example, by identifying genes that are mutated more frequently than expected from the background mutation rate (i.e., recurrence); by identifying

genes that exhibit other signals of positive selection across tumor samples (e.g., a high rate of non-silent mutations compared to silent mutations, or a bias towards the accumulation of functional mutations); by exploiting the tendency to sustain mutations in certain regions of the protein sequence based on the knowledge that whereas inactivating mutations are distributed along the sequence of the protein, gain-of-function mutations tend to occur specifically in particular residues or domains; or by exploiting the overrepresentation of mutations in specific functional residues, such as phosphorylation sites. Many of these mutations frequently occur in the functional regions of biologically active proteins (for example, kinase domains or binding domains) or interrupt active sites (for example, phosphorylation sites) resulting in loss-of-function or gain-of-function mutations, or they can occur in such a way that the three-dimensional structure and/or charge balance of the protein is perturbed sufficiently to interfere with normal function. Genomic analysis of large numbers of tumors reveals that mutations often occur at a limited number of amino acid positions. Therefore, a majority of the common mutations can be represented by a relatively small number of potential tumor-associated antigens or T cell epitopes.

[00251] A "recurrent cancer mutation" is a change in the amino acid sequence of a protein that occurs in multiple types of cancer and/or in multiple subjects having a particular types of cancer. Such mutations associated with a cancer can result in tumor-associated antigens that are not normally present in corresponding healthy tissue.

[00252] Tumor-driver genes and cancer-associated proteins having common mutations that occur across multiple cancers or among multiple cancer patients are known, and sequencing data across multiple tumor samples and multiple tumor types exists. *See, e.g.*, Chang et al. (2016) *Nat Biotechnol* 34(2):155-163; Tamborero et al. (2013) *Sci Rep* 3:2650, each of which is herein incorporated by reference in its entirety.

[00253] As another example, an antigenic peptide can be a heteroclitic antigenic peptide. For example, a heteroclitic antigenic peptide can be a fragment of a cancer-associated protein (i.e., a contiguous sequence of amino acids from a cancer-associated protein) comprising a heteroclitic mutation. A heteroclitic antigenic peptide can comprise a single heteroclitic mutation or can comprise two or more heteroclitic mutations (e.g., two heteroclitic mutations). The term "heteroclitic" refers to a peptide that generates an immune response that recognizes the native

peptide from which the heteroclitic peptide was derived (e.g., the peptide not containing the anchor residue mutations).

[00254] Some recombinant fusion polypeptides disclosed herein can comprise any combination of antigenic peptides comprising recurrent cancer mutations, antigenic peptides (e.g., from cancer-associated proteins) comprising heteroclitic mutations, and antigenic peptides (e.g., from cancer-associated proteins) expressed from minigene constructs (i.e., antigenic peptides such as a heteroclitic antigenic peptide fused to ubiquitin). For example, such a recombinant fusion polypeptide can comprise a PEST-containing peptide fused to two or more antigenic peptides, wherein at least one antigenic peptide is from a cancer-associated protein and comprises a recurrent cancer mutation, and at least one antigenic peptide is from a cancer-associated protein and comprises a heteroclitic mutation. Optionally, the PEST-containing peptide comprises a bacterial secretion signal sequence, and the fusion polypeptide further comprises a ubiquitin protein fused to a carboxy-terminal antigenic peptide, wherein the PEST-containing peptide, the two or more antigenic peptides, the ubiquitin, and the carboxy-terminal antigenic peptide are arranged in tandem from the amino-terminal end to the carboxy-terminal end of the fusion polypeptide.

[00255] Each antigenic peptide can also be hydrophilic or can score up to or below a certain hydropathy threshold, which can be predictive of secretability in *Listeria monocytogenes* or another bacteria of interest. For example, antigenic peptides can be scored by a Kyte and Doolittle hydropathy index 21 amino acid window, and all scoring above a cutoff (around 1.6) can be excluded as they are unlikely to be secretable by *Listeria monocytogenes*. Likewise, the combination of antigenic peptides or the fusion polypeptide can be hydrophilic or can score up to or below a certain hydropathy threshold, which can be predictive of secretability in *Listeria monocytogenes* or another bacteria of interest.

[00256] The antigenic peptides can be linked together in any manner. For example, the antigenic peptides can be fused directly to each other with no intervening sequence. Alternatively, the antigenic peptides can be linked to each other indirectly via one or more linkers, such as peptide linkers. In some cases, some pairs of adjacent antigenic peptides can be fused directly to each other, and other pairs of antigenic peptides can be linked to each other indirectly via one or more linkers. The same linker can be used between each pair of adjacent antigenic peptides, or any number of different linkers can be used between different pairs of

adjacent antigenic peptides. In addition, one linker can be used between a pair of adjacent antigenic peptides, or multiple linkers can be used between a pair of adjacent antigenic peptides.

[00257] Any suitable sequence can be used for a peptide linker. As an example, a linker sequence may be, for example, from 1 to about 50 amino acids in length. Some linkers may be hydrophilic. The linkers can serve varying purposes. For example, the linkers can serve to increase bacterial secretion, to facilitate antigen processing, to increase flexibility of the fusion polypeptide, to increase rigidity of the fusion polypeptide, or any other purpose. In some cases, different amino acid linker sequences are distributed between the antigenic peptides or different nucleic acids encoding the same amino acid linker sequence are distributed between the antigenic peptides (e.g., SEQ ID NOS: 84-94) in order to minimize repeats. This can also serve to reduce secondary structures, thereby allowing efficient transcription, translation, secretion, maintenance, or stabilization of the nucleic acid (e.g., plasmid) encoding the fusion polypeptide within a *Lm* recombinant vector strain population. Other suitable peptide linker sequences may be chosen, for example, based on one or more of the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the antigenic peptides; and (3) the lack of hydrophobic or charged residues that might react with the functional epitopes. For example, peptide linker sequences may contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al. (1985) *Gene* 40:39-46; Murphy et al. (1986) *Proc Natl Acad Sci USA* 83:8258-8262; US 4,935,233; and US 4,751,180, each of which is herein incorporated by reference in its entirety for all purposes. Specific examples of linkers include those in **Table 2** (each of which can be used by itself as a linker, in a linker comprising repeats of the sequence, or in a linker further comprising one or more of the other sequences in the table), although others can also be envisioned (*see, e.g.*, Reddy Chichili et al. (2013) *Protein Science* 22:153–167, herein incorporated by reference in its entirety for all purposes). Unless specified, "n" represents an undetermined number of repeats in the listed linker.

[00258] **Table 2. Linkers.**

Peptide Linker	Example	SEQ ID NO:	Hypothetical Purpose
$(GAS)_n$	GASGAS	33	Flexibility
$(GSA)_n$	GSAGSA	34	Flexibility
$(G)_n$; n = 4-8	GGGG	35	Flexibility
$(GGGGS)_n$; n = 1-3	GGGGS	36	Flexibility
VGKGGSGG	VGKGGSGG	37	Flexibility
$(PAPAP)_n$	PAPAP	38	Rigidity
$(EAAAK)_n$; n=1-3	EAAAK	39	Rigidity
$(AYL)_n$	AYLAYL	40	Antigen Processing
$(LRA)_n$	LRALRA	41	Antigen Processing
$(RLRA)_n$	RLRA	42	Antigen Processing

B. PEST-Containing Peptides

[00259] The recombinant fusion proteins disclosed herein comprise a PEST-containing peptide. The PEST-containing peptide may at the amino terminal (N-terminal) end of the fusion polypeptide (i.e., N-terminal to the antigenic peptides), may be at the carboxy terminal (C-terminal) end of the fusion polypeptide (i.e., C-terminal to the antigenic peptides), or may be embedded within the antigenic peptides. In some recombinant *Listeria* strains and methods, a PEST containing peptide is not part of and is separate from the fusion polypeptide. Fusion of an antigenic peptides to a PEST-like sequence, such as an LLO peptide, can enhance the immunogenicity of the antigenic peptides and can increase cell-mediated and antitumor immune responses (i.e., increase cell-mediated and anti-tumor immunity). *See, e.g.*, Singh et al. (2005) *J Immunol* 175(6):3663-3673, herein incorporated by reference in its entirety for all purposes.

[00260] A PEST-containing peptide is one that comprises a PEST sequence or a PEST-like sequence. PEST sequences in eukaryotic proteins have long been identified. For example, proteins containing amino acid sequences that are rich in prolines (P), glutamic acids (E), serines (S) and threonines (T) (PEST), generally, but not always, flanked by clusters containing several positively charged amino acids, have rapid intracellular half-lives (Rogers et al. (1986) *Science* 234:364-369, herein incorporated by reference in its entirety for all purposes). Further, it has been reported that these sequences target the protein to the ubiquitin-proteasome pathway for degradation (Rechsteiner and Rogers (1996) *Trends Biochem. Sci.* 21:267-271, herein incorporated by reference in its entirety for all purposes). This pathway is also used by eukaryotic cells to generate immunogenic peptides that bind to MHC class I and it has been hypothesized that PEST sequences are abundant among eukaryotic proteins that give rise to

immunogenic peptides (Realini et al. (1994) *FEBS Lett.* 348:109-113, herein incorporated by reference in its entirety for all purposes). Prokaryotic proteins do not normally contain PEST sequences because they do not have this enzymatic pathway. However, a PEST-like sequence rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T) has been reported at the amino terminus of LLO and has been reported to be essential for *L. monocytogenes* pathogenicity (Decatur and Portnoy (2000) *Science* 290:992-995, herein incorporated by reference in its entirety for all purposes). The presence of this PEST-like sequence in LLO targets the protein for destruction by proteolytic machinery of the host cell so that once the LLO has served its function and facilitated the escape of *L. monocytogenes* from the phagosomal or phagolysosomal vacuole, it is destroyed before it can damage the cells.

[00261] Identification of PEST and PEST-like sequences is well-known and is described, for example, in Rogers *et al.* (1986) *Science* 234(4774):364-378 and in Rechsteiner and Rogers (1996) *Trends Biochem. Sci.* 21:267-271, each of which is herein incorporated by reference in its entirety for all purposes. A PEST or PEST-like sequence can be identified using the PEST-find program. For example, a PEST-like sequence can be a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues. Optionally, the PEST-like sequence can be flanked by one or more clusters containing several positively charged amino acids. For example, a PEST-like sequence can be defined as a hydrophilic stretch of at least 12 amino acids in length with a high local concentration of proline (P), aspartate (D), glutamate (E), serine (S), and/or threonine (T) residues. In some cases, a PEST-like sequence contains no positively charged amino acids, namely arginine (R), histidine (H), and lysine (K). Some PEST-like sequences can contain one or more internal phosphorylation sites, and phosphorylation at these sites precedes protein degradation.

[00262] In one example, the PEST-like sequence fits an algorithm disclosed in Rogers et al. In another example, the PEST-like sequence fits an algorithm disclosed in Rechsteiner and Rogers. PEST-like sequences can also be identified by an initial scan for positively charged amino acids R, H, and K within the specified protein sequence. All amino acids between the positively charged flanks are counted, and only those motifs containing a number of amino acids equal to or higher than the window-size parameter are considered further. Optionally, a PEST-like sequence must contain at least one P, at least one D or E, and at least one S or T.

[00263] The quality of a PEST motif can be refined by means of a scoring parameter based on the local enrichment of critical amino acids as well as the motifs hydrophobicity. Enrichment of D, E, P, S, and T is expressed in mass percent (w/w) and corrected for one equivalent of D or E, one1 of P, and one of S or T. Calculation of hydrophobicity can also follow in principle the method of Kyte and Doolittle (1982) *J. Mol. Biol.* 157:105, herein incorporated by reference in its entirety for all purposes. For simplified calculations, Kyte-Doolittle hydropathy indices, which originally ranged from –4.5 for arginine to +4.5 for isoleucine, are converted to positive integers, using the following linear transformation, which yielded values from 0 for arginine to 90 for isoleucine: Hydropathy index = 10 * Kyte-Doolittle hydropathy index + 45.

[00264] A potential PEST motif's hydrophobicity can also be calculated as the sum over the products of mole percent and hydrophobicity index for each amino acid species. The desired PEST score is obtained as combination of local enrichment term and hydrophobicity term as expressed by the following equation: PEST score = 0.55 * DEPST - 0.5 * hydrophobicity index.

[00265] Thus, a PEST-containing peptide can refer to a peptide having a score of at least +5 using the above algorithm. Alternatively, it can refer to a peptide having a score of at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 32, at least 35, at least 38, at least 40, or at least 45.

[00266] Any other known available methods or algorithms can also be used to identify PEST-like sequences. *See, e.g.*, the CaSPredictor (Garay-Malpartida et al. (2005) *Bioinformatics* 21 Suppl 1:i169-76, herein incorporated by reference in its entirety for all purposes). Another method that can be used is the following: a PEST index is calculated for each stretch of appropriate length (e.g. a 30-35 amino acid stretch) by assigning a value of one to the amino acids Ser, Thr, Pro, Glu, Asp, Asn, or Gln. The coefficient value (CV) for each of the PEST residues is one and the CV for each of the other AA (non-PEST) is zero.

[00267] Examples of PEST-like amino acid sequences are those set forth in SEQ ID NOS: 43-51. One example of a PEST-like sequence is KENSISSMAPPASPPASPKTPIEKKHADEIDK (SEQ ID NO: 43). Another example of a PEST-like sequence is KENSISSMAPPASPPASPK (SEQ ID NO: 44). However, any PEST or PEST-like amino acid sequence can be used. PEST sequence peptides are known and are described, for example, in US 7,635,479; US 7,665,238;

and US 2014/0186387, each of which is herein incorporated by reference in its entirety for all purposes.

[00268] The PEST-like sequence can be from a *Listeria* species, such as from *Listeria monocytogenes*. For example, the *Listeria monocytogenes* ActA protein contains at least four such sequences (SEQ ID NOS: 45-48), any of which are suitable for use in the compositions and methods disclosed herein. Other similar PEST-like sequences include SEQ ID NOS: 52-54. Streptolysin O proteins from *Streptococcus* sp. also contain a PEST sequence. For example, *Streptococcus pyogenes* streptolysin O comprises the PEST sequence KQNTASTETTTTNEQPK (SEQ ID NO: 49) at amino acids 35-51 and *Streptococcus equisimilis* streptolysin O comprises the PEST-like sequence KQNTANTETTTTNEQPK (SEQ ID NO: 50) at amino acids 38-54. Another example of a PEST-like sequence is from *Listeria seeligeri* cytolysin, encoded by the *lso* gene: RSEVTISPAETPESPPATP (e.g., SEQ ID NO: 51).

[00269] Alternatively, the PEST-like sequence can be derived from other prokaryotic organisms. Other prokaryotic organisms wherein PEST-like amino acid sequences would be expected include, for example, other *Listeria* species.

(1) Listeriolysin O (LLO)

[00270] One example of a PEST-containing peptide that can be utilized in the compositions and methods disclosed herein is a listeriolysin O (LLO) peptide. An example of an LLO protein is the protein assigned GenBank Accession No. P13128 (SEQ ID NO: 55; nucleic acid sequence is set forth in GenBank Accession No. X15127). SEQ ID NO: 55 is a proprotein including a signal sequence. The first 25 amino acids of the proprotein is the signal sequence and is cleaved from LLO when it is secreted by the bacterium, thereby resulting in the full-length active LLO protein of 504 amino acids without the signal sequence. An LLO peptide disclosed herein can comprise the signal sequence or can comprise a peptide that does not include the signal sequence. Exemplary LLO proteins that can be used comprise, consist essentially of, or consist of the sequence set forth in SEQ ID NO: 55 or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms of SEQ ID NO: 55. Any sequence that encodes a fragment of an LLO protein or a homologue, variant, isoform, analog, fragment of a homologue, fragment of a variant, or fragment of an analog of an LLO protein can be used. A homologous LLO protein can have a

sequence identity with a reference LLO protein, for example, of greater than 70%, 72%, 75%, 78%, 80%, 82%, 83%, 85%, 87%, 88%, 90%, 92%, 93%, 95%, 96%, 97%, 98%, or 99%.

[00271] Another example of an LLO protein is set forth in SEQ ID NO: 56. LLO proteins that can be used can comprise, consist essentially of, or consist of the sequence set forth in SEQ ID NO: 56 or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms of SEQ ID NO: 56.

[00272] Another example of an LLO protein is an LLO protein from the *Listeria monocytogenes* 10403S strain, as set forth in GenBank Accession No.: ZP_01942330 or EBA21833, or as encoded by the nucleic acid sequence as set forth in GenBank Accession No.: NZ_AARZ01000015 or AARZ01000015.1. Another example of an LLO protein is an LLO protein from the *Listeria monocytogenes* 4b F2365 strain (*see, e.g.*, GenBank Accession No.: YP_012823), EGD-e strain (*see, e.g.*, GenBank Accession No.: NP_463733), or any other strain of *Listeria monocytogenes*. Yet another example of an LLO protein is an LLO protein from *Flavobacteriales* bacterium HTCC2170 (*see, e.g.*, GenBank Accession No.: ZP_01106747 or EAR01433, or encoded by GenBank Accession No.: NZ_AAOC01000003). LLO proteins that can be used can comprise, consist essentially of, or consist of any of the above LLO proteins or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms of the above LLO proteins.

[00273] Proteins that are homologous to LLO, or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms thereof, can also be used. One such example is alveolysin, which can be found, for example, in *Paenibacillus alvei* (*see, e.g.*, GenBank Accession No.: P23564 or AAA22224, or encoded by GenBank Accession No.: M62709). Other such homologous proteins are known.

[00274] The LLO peptide can be a full-length LLO protein or a truncated LLO protein or LLO fragment. Likewise, the LLO peptide can be one that retains one or more functionalities of a native LLO protein or lacks one or more functionalities of a native LLO protein. For example, the retained LLO functionality can be allowing a bacteria (e.g., *Listeria*) to escape from a phagosome or phagolysosome, or enhancing the immunogenicity of a peptide to which it is fused. The retained functionality can also be hemolytic function or antigenic function. Alternatively, the LLO peptide can be a non-hemolytic LLO. Other functions of LLO are known, as are methods and assays for evaluating LLO functionality.

[00275] An LLO fragment can be a PEST-like sequence or can comprise a PEST-like sequence. LLO fragments can comprise one or more of an internal deletion, a truncation from the C-terminal end, and a truncation from the N-terminal end. In some cases, an LLO fragment can comprise more than one internal deletion. Other LLO peptides can be full-length LLO proteins with one or more mutations.

[00276] Some LLO proteins or fragments have reduced hemolytic activity relative to wild type LLO or are non-hemolytic fragments. For example, an LLO protein can be rendered non-hemolytic by deletion or mutation of the activation domain at the carboxy terminus, by deletion or mutation of cysteine 484, or by deletion or mutation at another location.

[00277] Other LLO proteins are rendered non-hemolytic by a deletion or mutation of the cholesterol binding domain (CBD) as detailed in US 8,771,702, herein incorporated by reference in its entirety for all purposes. The mutations can comprise, for example, a substitution or a deletion. The entire CBD can be mutated, portions of the CBD can be mutated, or specific residues within the CBD can be mutated. For example, the LLO protein can comprise a mutation of one or more of residues C484, W491, and W492 (e.g., C484, W491, W492, C484 and W491, C484 and W492, W491 and W492, or all three residues) of SEQ ID NO: 55 or corresponding residues when optimally aligned with SEQ ID NO: 55 (e.g., a corresponding cysteine or tryptophan residue). As an example, a mutant LLO protein can be created wherein residues C484, W491, and W492 of LLO are substituted with alanine residues, which will substantially reduce hemolytic activity relative to wild type LLO. The mutant LLO protein with C484A, W491A, and W492A mutations is termed "mutLLO."

[00278] As another example, a mutant LLO protein can be created with an internal deletion comprising the cholesterol-binding domain. The sequence of the cholesterol-binding domain of SEQ ID NO: 55 set forth in SEQ ID NO: 74. For example, the internal deletion can be a 1-11 amino acid deletion, an 11-50 amino acid deletion, or longer. Likewise, the mutated region can be 1-11 amino acids, 11-50 amino acids, or longer (e.g., 1-50, 1-11, 2-11, 3-11, 4-11, 5-11, 6-11, 7-11, 8-11, 9-11, 10-11, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 12-50, 11-15, 11-20, 11-25, 11-30, 11-35, 11-40, 11-50, 11-60, 11-70, 11-80, 11-90, 11-100, 11-150, 15-20, 15-25, 15-30, 15-35, 15-40, 15-50, 15-60, 15-70, 15-80, 15-90, 15-100, 15-150, 20-25, 20-30, 20-35, 20-40, 20-50, 20-60, 20-70, 20-80, 20-90, 20-100, 20-150, 30-35, 30-40, 30-60, 30-70, 30-80, 30-90, 30-100, or 30-150 amino

acids). For example, a mutated region consisting of residues 470-500, 470-510, or 480-500 of SEQ ID NO: 55 will result in a deleted sequence comprising the CBD (residues 483-493 of SEQ ID NO: 55). However, the mutated region can also be a fragment of the CBD or can overlap with a portion of the CBD. For example, the mutated region can consist of residues 470-490, 480-488, 485-490, 486-488, 490-500, or 486-510 of SEQ ID NO: 55. For example, a fragment of the CBD (residues 484-492) can be replaced with a heterologous sequence, which will substantially reduce hemolytic activity relative to wild type LLO. For example, the CBD (ECTGLAWEWWR; SEQ ID NO: 74) can be replaced with a CTL epitope from the antigen NY-ESO-1 (ESLLMWITQCR; SEQ ID NO: 75), which contains the HLA-A2 restricted epitope 157-165 from NY-ESO-1. The resulting LLO is termed "ctLLO."

[00279] In some mutated LLO proteins, the mutated region can be replaced by a heterologous sequence. For example, the mutated region can be replaced by an equal number of heterologous amino acids, a smaller number of heterologous amino acids, or a larger number of amino acids (e.g., 1-50, 1-11, 2-11, 3-11, 4-11, 5-11, 6-11, 7-11, 8-11, 9-11, 10-11, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 12-50, 11-15, 11-20, 11-25, 11-30, 11-35, 11-40, 11-50, 11-60, 11-70, 11-80, 11-90, 11-100, 11-150, 15-20, 15-25, 15-30, 15-35, 15-40, 15-50, 15-60, 15-70, 15-80, 15-90, 15-100, 15-150, 20-25, 20-30, 20-35, 20-40, 20-50, 20-60, 20-70, 20-80, 20-90, 20-100, 20-150, 30-35, 30-40, 30-60, 30-70, 30-80, 30-90, 30-100, or 30-150 amino acids). Other mutated LLO proteins have one or more point mutations (e.g., a point mutation of 1 residue, 2 residues, 3 residues, or more). The mutated residues can be contiguous or not contiguous.

[00280] In one example embodiment, an LLO peptide may have a deletion in the signal sequence and a mutation or substitution in the CBD.

[00281] Some LLO peptides are N-terminal LLO fragments (i.e., LLO proteins with a C-terminal deletion). Some LLO peptides are at least 494, 489, 492, 493, 500, 505, 510, 515, 520, or 525 amino acids in length or 492-528 amino acids in length. For example, the LLO fragment can consist of about the first 440 or 441 amino acids of an LLO protein (e.g., the first 441 amino acids of SEQ ID NO: 55 or 56, or a corresponding fragment of another LLO protein when optimally aligned with SEQ ID NO: 55 or 56). Other N-terminal LLO fragments can consist of the first 420 amino acids of an LLO protein (e.g., the first 420 amino acids of SEQ ID NO: 55 or 56, or a corresponding fragment of another LLO protein when optimally aligned with SEQ ID

NO: 55 or 56). Other N-terminal fragments can consist of about amino acids 20-442 of an LLO protein (e.g., amino acids 20-442 of SEQ ID NO: 55 or 56, or a corresponding fragment of another LLO protein when optimally aligned with SEQ ID NO: 55 or 56). Other N-terminal LLO fragments comprise any ΔLLO without the activation domain comprising cysteine 484, and in particular without cysteine 484. For example, the N-terminal LLO fragment can correspond to the first 425, 400, 375, 350, 325, 300, 275, 250, 225, 200, 175, 150, 125, 100, 75, 50, or 25 amino acids of an LLO protein (e.g., the first 425, 400, 375, 350, 325, 300, 275, 250, 225, 200, 175, 150, 125, 100, 75, 50, or 25 amino acids of SEQ ID NO: 55 or 56, or a corresponding fragment of another LLO protein when optimally aligned with SEQ ID NO: 55 or 56). In some embodiments, the fragment comprises one or more PEST-like sequences. LLO fragments and truncated LLO proteins can contain residues of a homologous LLO protein that correspond to any one of the above specific amino acid ranges. The residue numbers need not correspond exactly with the residue numbers enumerated above (e.g., if the homologous LLO protein has an insertion or deletion relative to a specific LLO protein disclosed herein). Examples of N-terminal LLO fragments include SEQ ID NOS: 57, 58, and 59. LLO proteins that can be used comprise, consist essentially of, or consist of the sequence set forth in SEQ ID NO: 57, 58, or 59 or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms of SEQ ID NO: 57, 58, or 59. In some compositions and methods, the N-terminal LLO fragment set forth in SEQ ID NO: 59 is used. An example of a nucleic acid encoding the N-terminal LLO fragment set forth in SEQ ID NO: 59 is SEQ ID NO: 60.

(2) ActA

[00282] Another example of a PEST-containing peptide that can be utilized in the compositions and methods disclosed herein is an ActA peptide. ActA is a surface-associated protein and acts as a scaffold in infected host cells to facilitate the polymerization, assembly, and activation of host actin polymers in order to propel a *Listeria monocytogenes* through the cytoplasm. Shortly after entry into the mammalian cell cytosol, *L. monocytogenes* induces the polymerization of host actin filaments and uses the force generated by actin polymerization to move, first intracellularly and then from cell to cell. ActA is responsible for mediating actin nucleation and actin-based motility. The ActA protein provides multiple binding sites for host

cytoskeletal components, thereby acting as a scaffold to assemble the cellular actin polymerization machinery. The N-terminus of ActA binds to monomeric actin and acts as a constitutively active nucleation promoting factor by stimulating the intrinsic actin nucleation activity. The *actA* and *hly* genes are both members of the 10-kb gene cluster regulated by the transcriptional activator PrfA, and *actA* is upregulated approximately 226-fold in the mammalian cytosol. Any sequence that encodes an ActA protein or a homologue, variant, isoform, analog, fragment of a homologue, fragment of a variant, or fragment of an analog of an ActA protein can be used. A homologous ActA protein can have a sequence identity with a reference ActA protein, for example, of greater than 70%, 72%, 75%, 78%, 80%, 82%, 83%, 85%, 87%, 88%, 90%, 92%, 93%, 95%, 96%, 97%, 98%, or 99%.

[00283] One example of an ActA protein comprises, consists essentially of, or consists of the sequence set forth in SEQ ID NO: 61. Another example of an ActA protein comprises, consists essentially of, or consists of the sequence set forth in SEQ ID NO: 62. The first 29 amino acid of the proprotein corresponding to either of these sequences are the signal sequence and are cleaved from ActA protein when it is secreted by the bacterium. An ActA peptide can comprise the signal sequence (e.g., amino acids 1-29 of SEQ ID NO: 61 or 62), or can comprise a peptide that does not include the signal sequence. Other examples of ActA proteins comprise, consist essentially of, or consist of homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of isoforms, or fragments of analogs of SEQ ID NO: 61 or 62.

[00284] Another example of an ActA protein is an ActA protein from the *Listeria monocytogenes* 10403S strain (GenBank Accession No.: DQ054585) the NICPBP 54002 strain (GenBank Accession No.: EU394959), the S3 strain (GenBank Accession No.: EU394960), NCTC 5348 strain (GenBank Accession No.: EU394961), NICPBP 54006 strain (GenBank Accession No.: EU394962), M7 strain (GenBank Accession No.: EU394963), S19 strain (GenBank Accession No.: EU394964), or any other strain of *Listeria monocytogenes*. LLO proteins that can be used can comprise, consist essentially of, or consist of any of the above LLO proteins or homologues, variants, isoforms, analogs, fragments, fragments of homologues, fragments of variants, fragments of analogs, and fragments of isoforms of the above LLO proteins.

[00285] ActA peptides can be full-length ActA proteins or truncated ActA proteins or ActA fragments (e.g., N-terminal ActA fragments in which a C-terminal portion is removed). In some

embodiments, truncated ActA proteins comprise at least one PEST sequence (e.g., more than one PEST sequence). In addition, truncated ActA proteins can optionally comprise an ActA signal peptide. Examples of PEST-like sequences contained in truncated ActA proteins include SEQ ID NOS: 45-48. Some such truncated ActA proteins comprise at least two of the PEST-like sequences set forth in SEQ ID NOS: 45-48 or homologs thereof, at least three of the PEST-like sequences set forth in SEQ ID NOS: 45-48 or homologs thereof, or all four of the PEST-like sequences set forth in SEQ ID NOS: 45-48 or homologs thereof. Examples of truncated ActA proteins include those comprising, consisting essentially of, or consisting of about residues 30-122, about residues 30-229, about residues 30-332, about residues 30-200, or about residues 30-399 of a full length ActA protein sequence (e.g., SEQ ID NO: 62). Other examples of truncated ActA proteins include those comprising, consisting essentially of, or consisting of about the first 50, 100, 150, 200, 233, 250, 300, 390, 400, or 418 residues of a full length ActA protein sequence (e.g., SEQ ID NO: 62). Other examples of truncated ActA proteins include those comprising, consisting essentially of, or consisting of about residues 200-300 or residues 300-400 of a full length ActA protein sequence (e.g., SEQ ID NO: 62). For example, the truncated ActA consists of the first 390 amino acids of the wild type ActA protein as described in US 7,655,238, herein incorporated by reference in its entirety for all purposes. As another example, the truncated ActA can be an ActA-N100 or a modified version thereof (referred to as ActA-N100*) in which a PEST motif has been deleted and containing the nonconservative QDNKR (SEQ ID NO: 73) substitution as described in US 2014/0186387, herein incorporated by references in its entirety for all purposes. Alternatively, truncated ActA proteins can contain residues of a homologous ActA protein that corresponds to one of the above amino acid ranges or the amino acid ranges of any of the ActA peptides disclosed herein. The residue numbers need not correspond exactly with the residue numbers enumerated herein (e.g., if the homologous ActA protein has an insertion or deletion, relative to an ActA protein utilized herein, then the residue numbers can be adjusted accordingly).

[00286] Examples of truncated ActA proteins include, for example, proteins comprising, consisting essentially of, or consisting of the sequence set forth in SEQ ID NO: 63, 64, 65, or 66 or homologues, variants, isoforms, analogs, fragments of variants, fragments of isoforms, or fragments of analogs of SEQ ID NO: 63, 64, 65, or 66. SEQ ID NO: 63 referred to as ActA/PEST1 and consists of amino acids 30-122 of the full length ActA sequence set forth in

SEQ ID NO: 62. SEQ ID NO: 64 is referred to as ActA/PEST2 or LA229 and consists of amino acids 30-229 of the full length ActA sequence set forth in the full-length ActA sequence set forth in SEQ ID NO: 62. SEQ ID NO: 65 is referred to as ActA/PEST3 and consists of amino acids 30-332 of the full-length ActA sequence set forth in SEQ ID NO: 62. SEQ ID NO: 66 is referred to as ActA/PEST4 and consists of amino acids 30-399 of the full-length ActA sequence set forth in SEQ ID NO: 62. As a specific example, the truncated ActA protein consisting of the sequence set forth in SEQ ID NO: 64 can be used.

[00287] Examples of truncated ActA proteins include, for example, proteins comprising, consisting essentially of, or consisting of the sequence set forth in SEQ ID NO: 67, 69, 70, or 72 or homologues, variants, isoforms, analogs, fragments of variants, fragments of isoforms, or fragments of analogs of SEQ ID NO: 67, 69, 70, or 72. As a specific example, the truncated ActA protein consisting of the sequence set forth in SEQ ID NO: 67 (encoded by the nucleic acid set forth in SEQ ID NO: 68) can be used. As another specific example, the truncated ActA protein consisting of the sequence set forth in SEQ ID NO: 70 (encoded by the nucleic acid set forth in SEQ ID NO: 71) can be used. SEQ ID NO: 71 is the first 1170 nucleotides encoding ActA in the *Listeria monocytogenes* 10403S strain. In some cases, the ActA fragment can be fused to a heterologous signal peptide. For example, SEQ ID NO: 72 sets forth an ActA fragment fused to an Hly signal peptide.

C. Generating Immunotherapy Constructs Encoding Recombinant Fusion Polypeptides

[00288] Also provided herein are methods for generating immunotherapy constructs encoding or compositions comprising the recombinant fusion polypeptides disclosed herein. For example, such methods can comprise selecting and designing antigenic peptides to include in the immunotherapy construct (and, for example, testing the hydropathy of the each antigenic peptide, and modifying or deselecting an antigenic peptide if it scores above a selected hydropathy index threshold value), designing one or more fusion polypeptides comprising each of the selected antigenic peptides, and generating a nucleic acid construct encoding the fusion polypeptide.

[00289] The antigenic peptides can be screened for hydrophobicity or hydrophilicity. Antigenic peptides can be selected, for example, if they are hydrophilic or if they score up to or

below a certain hydropathy threshold, which can be predictive of secretability in a particular bacteria of interest (e.g., *Listeria monocytogenes*). For example, antigenic peptides can be scored by Kyte and Doolittle hydropathy index with a 21 amino acid window, all scoring above cutoff (around 1.6) are excluded as they are unlikely to be secretable by *Listeria monocytogenes*. *See, e.g.*, Kyte-Doolittle (1982) *J Mol Biol* 157(1):105–132; herein incorporated by reference in its entirety for all purposes. Alternatively, an antigenic peptide scoring about a selected cutoff can be altered (e.g., changing the length of the antigenic peptide). Other sliding window sizes that can be used include, for example, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or more amino acids. For example, the sliding window size can be 9-11 amino acids, 11-13 amino acids, 13-15 amino acids, 15-17 amino acids, 17-19 amino acids, 19-21 amino acids, 21-23 amino acids, 23-25 amino acids, or 25-27 amino acids. Other cutoffs that can be used include, for example, the following ranges 1.2-1.4, 1.4-1.6, 1.6-1.8, 1.8-2.0, 2.0-2.2 2.2-2.5, 2.5-3.0, 3.0-3.5, 3.5-4.0, or 4.0-4.5, or the cutoff can be 1.4, 1.5, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.3, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, or 4.5. The cutoff can vary, for example, depending on the genus or species of the bacteria being used to deliver the fusion polypeptide.

[00290] Other suitable hydropathy plots or other appropriate scales include, for example, those reported in Rose et al. (1993) *Annu Rev Biomol Struct* 22:381–415; Biswas et al. (2003) *Journal of Chromatography A* 1000:637–655; Eisenberg (1984) *Ann Rev Biochem* 53:595–623; Abraham and Leo (1987) *Proteins: Structure, Function and Genetics* 2:130-152; Sweet and Eisenberg (1983) *Mol Biol* 171:479-488; Bull and Breese (1974) *Arch Biochem Biophys* 161:665-670; Guy (1985) *Biophys J* 47:61-70; Miyazawa et al. (1985) *Macromolecules* 18:534-552; Roseman (1988) *J Mol Biol* 200:513-522; Wolfenden et al. (1981) *Biochemistry* 20:849-855; Wilson (1981) *Biochem J* 199:31-41; Cowan and Whittaker (1990) *Peptide Research* 3:75-80; Aboderin (1971) *Int J Biochem* 2:537-544; Eisenberg et al. (1984) *J Mol Biol* 179:125-142; Hopp and Woods (1981) *Proc Natl Acad Sci USA* 78:3824-3828; Manavalan and Ponnuswamy (1978) *Nature* 275:673-674; Black and Mould (1991) *Anal Biochem* 193:72-82; Fauchere and Pliska (1983) *Eur J Med Chem* 18:369-375; Janin (1979) *Nature* 277:491-492; Rao and Argos (1986) *Biochim Biophys Acta* 869:197-214; Tanford (1962) *Am Chem Soc* 84:4240-4274; Welling et al. (1985) *FEBS Lett* 188:215-218; Parker et al. (1986) *Biochemistry* 25:5425-5431;

and Cowan and Whittaker (1990) *Peptide Research* 3:75-80, each of which is herein incorporated by reference in its entirety for all purposes.

[00291] Optionally, the antigenic peptides can be scored for their ability to bind to the subject human leukocyte antigen (HLA) type (for example by using the Immune Epitope Database (IED) available at www.iedb.org, which includes netMHCpan, ANN, SMMPMBEC. SMM, CombLib_Sidney2008, PickPocket, and netMHCcons) and ranked by best MHC binding score from each antigenic peptide. Other sources include TEpredict (tepredict.sourceforge.net/help.html) or other available MHC binding measurement scales. Cutoffs may be different for different expression vectors such as *Salmonella*.

[00292] Optionally, the antigenic peptides can be screened for immunosuppressive epitopes (e.g., T-reg epitopes, IL-10-inducing T helper epitopes, and so forth) to deselect antigenic peptides or to avoid immunosuppressive influences.

[00293] Optionally, a predicative algorithm for immunogenicity of the epitopes can be used to screen the antigenic peptides. However, these algorithms are at best 20% accurate in predicting which peptide will generate a T cell response. Alternatively, no screening/predictive algorithms are used. Alternatively, the antigenic peptides can be screened for immunogenicity. For example, this can comprise contacting one or more T cells with an antigenic peptide, and analyzing for an immunogenic T cell response, wherein an immunogenic T cell response identifies the peptide as an immunogenic peptide. This can also comprise using an immunogenic assay to measure secretion of at least one of CD25, CD44, or CD69 or to measure secretion of a cytokine selected from the group comprising IFN-γ, TNF-α, IL-1, and IL-2 upon contacting the one or more T cells with the peptide, wherein increased secretion identifies the peptide as comprising one or more T cell epitopes.

[00294] The selected antigenic peptides can be arranged into one or more candidate orders for a potential fusion polypeptide. If there are more usable antigenic peptides than can fit into a single plasmid, different antigenic peptides can be assigned priority ranks as needed/desired and/or split up into different fusion polypeptides (e.g., for inclusion in different recombinant *Listeria* strains). Priority rank can be determined by factors such as relative size, priority of transcription, and/or overall hydrophobicity of the translated polypeptide. The antigenic peptides can be arranged so that they are joined directly together without linkers, or any combination of linkers between any number of pairs of antigenic peptides, as disclosed in more detail elsewhere

herein. The number of linear antigenic peptides to be included can be determined based on consideration of the number of constructs needed versus the mutational burden, the efficiency of translation and secretion of multiple epitopes from a single plasmid, and the MOI needed for each bacteria or *Lm* comprising a plasmid.

[00295] The combination of antigenic peptides or the entire fusion polypeptide (i.e., comprising the antigenic peptides and the PEST-containing peptide and any tags) also be scored for hydrophobicity. For example, the entirety of the fused antigenic peptides or the entire fusion polypeptide can be scored for hydropathy by a Kyte and Doolittle hydropathy index with a sliding 21 amino acid window. If any region scores above a cutoff (e.g., around 1.6), the antigenic peptides can be reordered or shuffled within the fusion polypeptide until an acceptable order of antigenic peptides is found (i.e., one in which no region scores above the cutoff). Alternatively, any problematic antigenic peptides can be removed or redesigned to be of a different size. Alternatively or additionally, one or more linkers between antigenic peptides as disclosed elsewhere herein can be added or modified to change the hydrophobicity. As with hydropathy testing for the individual antigenic peptides, other window sizes can be used, or other cutoffs can be used (e.g., depending on the genus or species of the bacteria being used to deliver the fusion polypeptide). In addition, other suitable hydropathy plots or other appropriate scales could be used.

[00296] Optionally, the combination of antigenic peptides or the entire fusion polypeptide can be further screened for immunosuppressive epitopes (e.g., T-reg epitopes, IL-10-inducing T helper epitopes, and so forth) to deselect antigenic peptides or to avoid immunosuppressive influences.

[00297] A nucleic acid encoding a candidate combination of antigenic peptides or fusion polypeptide can then be designed and optimized. For example, the sequence can be optimized for increased levels of translation, duration of expression, levels of secretion, levels of transcription, and any combination thereof. For example, the increase can be 2-fold to 1000-fold, 2-fold to 500-fold, 2-fold to 100-fold, 2-fold to 50-fold, 2-fold to 20-fold, 2-fold to 10-fold, or 3-fold to 5-fold relative to a control, non-optimized sequence.

[00298] For example, the fusion polypeptide or nucleic acid encoding the fusion polypeptide can be optimized for decreased levels of secondary structures possibly formed in the oligonucleotide sequence, or alternatively optimized to prevent attachment of any enzyme that

may modify the sequence. Expression in bacterial cells can be hampered, for example, by transcriptional silencing, low mRNA half-life, secondary structure formation, attachment sites of oligonucleotide binding molecules such as repressors and inhibitors, and availability of rare tRNAs pools. The source of many problems in bacterial expressions is found within the original sequence. The optimization of RNAs may include modification of cis acting elements, adaptation of its GC-content, modifying codon bias with respect to non-limiting tRNAs pools of the bacterial cell, and avoiding internal homologous regions. Thus, optimizing a sequence can entail, for example, adjusting regions of very high (> 80%) or very low (< 30%) GC content. Optimizing a sequence can also entail, for example, avoiding one or more of the following cis-acting sequence motifs: internal TATA-boxes, chi-sites, and ribosomal entry sites; AT-rich or GC-rich sequence stretches; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites; branch points; or a combination thereof. Optimizing expression can also entail adding sequence elements to flanking regions of a gene and/or elsewhere in the plasmid.

[00299] Optimizing a sequence can also entail, for example, adapting the codon usage to the codon bias of host genes (e.g., *Listeria monocytogenes* genes). For example, the codons that can be used for *Listeria monocytogenes* include A = GCA, G = GGT, L = TTA, Q = CAA, V = GTT, C = TGT, H = CAT, M = ATG, R = CGT, W = TGG, D = GAT, I = ATT, N = AAC, S = TCT, Y = TAT, E = GAA, K = AAA, P = CCA, T = ACA, F = TTC, and STOP = TAA.

[00300] A nucleic acid encoding a fusion polypeptide can be generated and introduced into a delivery vehicle such as a bacteria strain or *Listeria* strain. Other delivery vehicles may be suitable for DNA immunotherapy or peptide immunotherapy, such as a vaccinia virus or virus-like particle. Once a plasmid encoding a fusion polypeptide is generated and introduced into a bacteria strain or *Listeria* strain, the bacteria or *Listeria* strain can be cultured and characterized to confirm expression and secretion of the fusion polypeptide comprising the antigenic peptides.

V. Immunogenic Compositions, Pharmaceutical Compositions, and Vaccines

[00301] Also provided are immunogenic compositions, pharmaceutical compositions, or vaccines comprising a lyophilized recombinant bacteria or *Listeria* strain as disclosed herein, optionally wherein the lyophilized recombinant bacteria or *Listeria* strain is reconstituted by dissolving in an amount of solvent. An immunogenic composition comprising a *Listeria* strain

can be inherently immunogenic by virtue of its comprising a *Listeria* strain and/or the composition can also further comprise an adjuvant. Other immunogenic compositions comprise DNA immunotherapy or peptide immunotherapy compositions.

[00302] The term "immunogenic composition" refers to any composition containing an antigen that elicits an immune response against the antigen in a subject upon exposure to the composition. The immune response elicited by an immunogenic composition can be to a particular antigen or to a particular epitope on the antigen.

[00303] An immunogenic composition can comprise a single lyophilized or reconstituted recombinant bacteria or *Listeria* strain as disclosed herein, or it can comprise multiple different lyophilized or reconstituted recombinant bacteria or *Listeria* strains as disclosed herein. A bacteria or *Listeria* strain comprising a first recombinant fusion polypeptide is different from a bacteria or *Listeria* strain comprising a second recombinant fusion polypeptide, for example, if the first recombinant fusion polypeptide includes one antigenic peptide that the second recombinant fusion polypeptide does not. The two recombinant fusion polypeptides can include some of the same antigenic peptides and still be considered different. Such different lyophilized or reconstituted recombinant bacteria or *Listeria* strains can be administered concomitantly to a subject or sequentially to a subject. Sequential administration can be particularly useful when a drug substance comprising a lyophilized or reconstituted recombinant *Listeria* strain (or recombinant fusion polypeptide or nucleic acid) disclosed herein is in different dosage forms and/or is administered on different dosing schedules (e.g., one composition from the mixture is administered at least daily and another is administered less frequently, such as once weekly, once every two weeks, or once every three weeks). The multiple lyophilized or reconstituted recombinant bacteria or *Listeria* strains can each comprise a different set of antigenic peptides. Alternatively, two or more of the lyophilized or reconstituted recombinant bacteria or *Listeria* strains can comprise the same set of antigenic peptides (e.g., the same set of antigenic peptides in a different order).

[00304] An immunogenic composition can additionally comprise an adjuvant (e.g., two or more adjuvants), a cytokine, a chemokine, or combination thereof. Optionally, an immunogenic composition can additionally comprises antigen presenting cells (APCs), which can be autologous or can be allogeneic to the subject.

[00305] The term adjuvant includes compounds or mixtures that enhance the immune response to an antigen. For example, an adjuvant can be a non-specific stimulator of an immune response or substances that allow generation of a depot in a subject which when combined with an immunogenic composition disclosed herein provides for an even more enhanced and/or prolonged immune response. An adjuvant can favor, for example, a predominantly Th1-mediated immune response, a Th1-type immune response, or a Th1-mediated immune response. Likewise, an adjuvant can favor a cell-mediated immune response over an antibody-mediated response. Alternatively, an adjuvant can favor an antibody-mediated response. Some adjuvants can enhance the immune response by slowly releasing the antigen, while other adjuvants can mediate their effects by any of the following mechanisms: increasing cellular infiltration, inflammation, and trafficking to the injection site, particularly for antigen-presenting cells (APC); promoting the activation state of APCs by upregulating costimulatory signals or major histocompatibility complex (MHC) expression; enhancing antigen presentation; or inducing cytokine release for indirect effect.

[00306] Examples of adjuvants include saponin QS21, CpG oligonucleotides, unmethylated CpG-containing oligonucleotides, MPL, TLR agonists, TLR4 agonists, TLR9 agonists, Resiquimod®, imiquimod, cytokines or nucleic acids encoding the same, chemokines or nucleic acids encoding same, IL-12 or a nucleic acid encoding the same, IL-6 or a nucleic acid encoding the same, and lipopolysaccharides. Another example of a suitable adjuvant is Montanide ISA 51. Montanide ISA 51 contains a natural metabolizable oil and a refined emulsifier. Other examples of a suitable adjuvant include granulocyte/macrophage colony-stimulating factor (GM-CSF) or a nucleic acid encoding the same and keyhole limpet hemocyanin (KLH) proteins or nucleic acids encoding the same. The GM-CSF can be, for example, a human protein grown in a yeast (*S. cerevisiae*) vector. GM-CSF promotes clonal expansion and differentiation of hematopoietic progenitor cells, antigen presenting cells (APCs), dendritic cells, and T cells.

[00307] Yet another example of a suitable adjuvant is detoxified listeriolysin O (dtLLO) protein. Detoxification can be accomplished by introducing point mutations for three selected amino acids important for binding of LLO to cholesterol and for eventual membrane pore formation. The three targeted amino acids are present in the cholesterol binding domain of LLO (ECTGLAWEWWR; SEQ ID NO: 74) and can be modified in the sequence (EATGLAWEAAR; SEQ ID NO: 96) by point mutations introduced into the DNA sequence by PCR. One example

of a dtLLO suitable for use as an adjuvant is encoded by SEQ ID NO: 95. The detoxified, nonhemolytic form of LLO (dtLLO) is an effective adjuvant in tumor immunotherapy and may activate innate and cellular immune responses by acting as a PAMP. A dtLLO encoded by a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 95 is also suitable for use as an adjuvant.

[00308] Yet other examples of adjuvants include growth factors or nucleic acids encoding the same, cell populations, Freund's incomplete adjuvant, aluminum phosphate, aluminum hydroxide, BCG (bacille Calmette-Guerin), alum, interleukins or nucleic acids encoding the same, quill glycosides, monophosphoryl lipid A, liposomes, bacterial mitogens, bacterial toxins, or any other type of known adjuvant (*see, e.g.*, Fundamental Immunology, 5th ed. (August 2003): William E. Paul (Editor); Lippincott Williams & Wilkins Publishers; Chapter 43: Vaccines, GJV Nossal, which is herein incorporated by reference in its entirety for all purposes).

[00309] An immunogenic composition can further comprise one or more immunomodulatory molecules. Examples include interferon gamma, a cytokine, a chemokine, and a T cell stimulant.

[00310] An immunogenic composition can be in the form of a vaccine or pharmaceutical composition. The terms "vaccine" and "pharmaceutical composition" are interchangeable and refer to an immunogenic composition in a pharmaceutically acceptable carrier for *in vivo* administration to a subject. A vaccine may be, for example, a vaccine contained within and delivered by a cell (e.g., a recombinant *Listeria* as disclosed herein). A vaccine may prevent a subject from contracting or developing a disease and/or a vaccine may be therapeutic to a subject having a disease.

[00311] A "pharmaceutically acceptable carrier" refers to a vehicle for containing an immunogenic composition that can be introduced into a subject without significant adverse effects and without having deleterious effects on the immunogenic composition. That is, "pharmaceutically acceptable" refers to any formulation which is safe, and provides the appropriate delivery for the desired route of administration of an effective amount of at least one immunogenic composition for use in the methods disclosed herein. Pharmaceutically acceptable carriers or vehicles or excipients are well-known. Descriptions of suitable pharmaceutically acceptable carriers, and factors involved in their selection, are found in a variety of readily available sources such as, for example, *Remington's Pharmaceutical Sciences,* 18th ed., 1990, herein incorporated by reference in its entirety for all purposes. Such carriers can be suitable for

any route of administration (e.g., parenteral, enteral (e.g., oral), or topical application). Such pharmaceutical compositions can be buffered, for example, wherein the pH is maintained at a particular desired value, ranging from pH 4.0 to pH 9.0, in accordance with the stability of the immunogenic compositions and route of administration.

[00312] Suitable pharmaceutically acceptable carriers include, for example, sterile water, salt solutions such as saline, glucose, buffered solutions such as phosphate buffered solutions or bicarbonate buffered solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates (e.g., lactose, amylose or starch), magnesium stearate, talc, silicic acid, viscous paraffin, white paraffin, glycerol, alginates, hyaluronic acid, collagen, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, and the like. Pharmaceutical compositions or vaccines may also include auxiliary agents including, for example, diluents, stabilizers (e.g., sugars and amino acids), preservatives, wetting agents, emulsifiers, pH buffering agents, viscosity enhancing additives, lubricants, salts for influencing osmotic pressure, buffers, vitamins, coloring, flavoring, aromatic substances, and the like which do not deleteriously react with the immunogenic composition.

[00313] For liquid formulations (e.g., in embodiments wherein the lyophilized recombinant bacteria or *Listeria* strain is reconstituted by dissolving in an amount of solvent), for example, pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, emulsions, or oils. Non-aqueous solvents include, for example, propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include, for example, water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Examples of oils include those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, olive oil, sunflower oil, and fish-liver oil. Solid carriers/diluents include, for example, a gum, a starch (e.g., corn starch, pregelatinized starch), a sugar (e.g., lactose, mannitol, sucrose, or dextrose), a cellulosic material (e.g., microcrystalline cellulose), an acrylate (e.g., polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof.

[00314] Optionally, sustained or directed release pharmaceutical compositions or vaccines can be formulated. This can be accomplished, for example, through use of liposomes or compositions wherein the active compound is protected with differentially degradable coatings

(e.g., by microencapsulation, multiple coatings, and so forth). Such compositions may be formulated for immediate or slow release. It is also possible to freeze-dry the compositions and use the lyophilisates obtained (e.g., for the preparation of products for injection).

[00315] An immunogenic composition, pharmaceutical composition, or vaccine disclosed herein may also comprise one or more additional compounds effective in preventing or treating cancer. For example, the additional compound may comprise a compound useful in chemotherapy, such as amsacrine, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clofarabine, crisantaspase, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, fludarabine, fluorouracil (5-FU), gemcitabine, gliadelimplants, hydroxycarbamide, idarubicin, ifosfamide, irinotecan, leucovorin, liposomaldoxorubicin, liposomaldaunorubicin, lomustine, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitoxantrone, oxaliplatin, paclitaxel (Taxol), pemetrexed, pentostatin, procarbazine, raltitrexed, satraplatin, streptozocin, tegafur-uracil, temozolomide, teniposide, thiotepa, tioguanine, topotecan, treosulfan, vinblastine, vincristine, vindesine, vinorelbine, or a combination thereof. The additional compound can also comprise other biologics, including Herceptin® (trastuzumab) against the HER2 antigen, Avastin® (bevacizumab) against VEGF, or antibodies to the EGF receptor, such as Erbitux® (cetuximab), and Vectibix® (panitumumab). The additional compound can also comprise, for example, an additional immunotherapy.

[00316] An additional compound can also comprise an immune checkpoint inhibitor antagonist, such as a PD-1 signaling pathway inhibitor, a CD-80/86 and CTLA-4 signaling pathway inhibitor, a T cell membrane protein 3 (TIM3) signaling pathway inhibitor, an adenosine A2a receptor (A2aR) signaling pathway inhibitor, a lymphocyte activation gene 3 (LAG3) signaling pathway inhibitor, a killer immunoglobulin receptor (KIR) signaling pathway inhibitor, a CD40 signaling pathway inhibitor, or any other antigen-presenting cell/T cell signaling pathway inhibitor. Examples of immune checkpoint inhibitor antagonists include an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof. An additional compound can also comprise a T cell stimulator, such as an antibody or functional fragment thereof binding to a T-cell receptor co-stimulatory molecule, an antigen presenting cell receptor binding co-stimulatory molecule, or a member of the TNF receptor superfamily. The T-

cell receptor co-stimulatory molecule can comprise, for example, CD28 or ICOS. The antigen presenting cell receptor binding co-stimulatory molecule can comprise, for example, a CD80 receptor, a CD86 receptor, or a CD46 receptor. The TNF receptor superfamily member can comprise, for example, glucocorticoid-induced TNF receptor (GITR), OX40 (CD134 receptor), 4-1BB (CD137 receptor), or TNFR25. *See, e.g.*, WO2016100929, WO2016011362, and WO2016011357, each of which is incorporated by reference in its entirety for all purposes.

VI. Therapeutic Methods

[00317] The lyophilized bacteria or *Listeria* strains (optionally wherein the lyophilized recombinant bacteria or *Listeria* strain is reconstituted by dissolving in an amount of solvent), immunogenic compositions, pharmaceutical compositions, and vaccines disclosed herein can be used in various methods. For example, they can be used in methods of inducing or enhancing an anti-disease-associated-antigen (e.g., cancer-associated antigen or tumor-associated antigen) immune response in a subject, in methods of inducing or enhancing an anti-disease (e.g., anti-tumor or anti-cancer) immune response in a subject, in methods of treating a disease (e.g., a tumor or cancer) in a subject, in methods of preventing a disease (e.g., tumor or cancer) in a subject, or in methods of protecting a subject against a disease (e.g., tumor or cancer). They can also be used in methods of increasing the ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, wherein the T effector cells are targeted to a disease-associated antigen. They can also be used in methods for increasing disease-associated-antigen antigen T cells in a subject, increasing survival time of a subject having a disease, delaying the onset of a disease in a subject, or alleviating symptoms of a disease in a subject.

[00318] A method of inducing or enhancing an anti-disease-associated antigen immune response in a subject can comprise, for example, administering to the subject a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein. An anti-disease-associated antigen immune response can thereby be induced or enhanced in the subject. For example, in the case of a lyophilized or reconstituted recombinant *Listeria* strain, the *Listeria* strain can express the fusion polypeptide, thereby eliciting an immune response in the subject. The immune response can comprise, for example, a T-cell response, such as a CD4+FoxP3- T cell response, a CD8+ T cell response, or a CD4+FoxP3- and CD8+ T cell response. Such methods can also increase the

ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor microenvironments of the subject, allowing for a more profound anti-tumor response in the subject.

[00319] A method of inducing or enhancing an anti-disease (e.g., anti-cancer or anti-tumor) immune response in a subject can comprise, for example, administering to the subject a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein. An anti-disease immune response can thereby be induced or enhanced in the subject. For example, in the case of a recombinant *Listeria* strain, the *Listeria* strain can express the fusion polypeptide, thereby eliciting an anti-disease response in the subject.

[00320] A method of treating a disease (e.g., cancer or tumor) in a subject, can comprise, for example, administering to the subject a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein. The subject can then mount an immune response against the disease expressing the disease-associated antigen, thereby treating the disease in the subject.

[00321] A method of preventing a disease (e.g., tumor or cancer) in a subject or protecting a subject against developing a disease, can comprise, for example, administering to the subject a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein. The subject can then mount an immune response against the disease-associated antigen, thereby preventing a disease or protecting the subject against developing a disease.

[00322] In some of the above methods, two or more lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines are administered. The multiple recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines can be administered sequentially in any order or combination, or can be administered simultaneously in any combination. As an example, if four different *Listeria* strains are being administered, they can be administered sequentially, they can be administered simultaneously, or they can be administered in any combination (e.g., administering the first and second strains simultaneously and subsequently administering the third and fourth strains simultaneously). Optionally, in the case of sequential administration, the compositions can be administered during the same immune response. In some embodiments the compositions are administered within 0-10 or 3-7 days of each other. The

multiple recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines can each comprise a different set of antigenic peptides. Alternatively, two or more can comprise the same set of antigenic peptides (e.g., the same set of antigenic peptides in a different order).

[00323] In some methods, the disease is a cancer or tumor. Cancer is a physiological condition in mammals that is typically characterized by unregulated cell growth and proliferation. Cancers can be hematopoietic malignancies or solid tumors (i.e., masses of cells that result from excessive cell growth or proliferation, including pre-cancerous legions). Metastatic cancer refers to a cancer that has spread from the place where it first started to another place in the body. Tumors formed by metastatic cancer cells are called a metastatic tumor or a metastasis, which is a term also used to refer to the process by which cancer cells spread to other parts of the body. In general, metastatic cancer has the same name and same type of cancer cells as the original, or primary, cancer. Examples of solid tumors include melanoma, carcinoma, blastoma, and sarcoma. Hematologic malignancies include, for example, leukemia or lymphoid malignancies, such as lymphoma. Exemplary categories of cancers include brain, breast, gastrointestinal, genitourinary, gynecologic, head and neck, heme, skin and thoracic. Brain malignancies include, for example, glioblastoma, high-grade pontine glioma, low-grade glioma, medulloblastoma, neuroblastoma, and pilocytic astrocytoma. Gastrointestinal cancers include, for example, colorectal, gallbladder, hepatocellular, pancreas, PNET, gastric, and esophageal. Genitourinary cancers include, for example, adrenocortical, bladder, kidney chromophobe, renal (clear cell), renal (papillary), rhabdoid cancers, and prostate. Gynecologic cancers include, for example, uterine carcinosarcoma, uterine endometrial, serous ovarian, and cervical. Head and neck cancers include, for example, thyroid, nasopharyngeal, head and neck, and adenoid cystic. Heme cancers include, for example, multiple myeloma, myelodysplasia, mantle-cell lymphoma, acute lymphoblastic leukemia (ALL), non-lymphoma, chronic lymphocytic leukemia (CLL), and acute myeloid leukemia (AML). Skin cancers includes, for example, cutaneous melanoma and squamous cell carcinoma. Thoracic cancers include, for example, squamous lung, small-cell lung, and lung adenocarcinoma.

[00324] More particular examples of such cancers include squamous cell cancer or carcinoma (e.g., oral squamous cell carcinoma), myeloma, oral cancer, juvenile nasopharyngeal angiofibroma, neuroendocrine tumors, lung cancer, cancer of the peritoneum, hepatocellular

cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioma, glioblastoma, glial tumors, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, hepatocellular carcinoma, breast cancer, triple-negative breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial cancer or uterine cancer or carcinoma, salivary gland carcinoma, kidney or renal cancer (e.g., renal cell carcinoma), prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, fibrosarcoma, gallbladder cancer, osteosarcoma, mesothelioma, as well as head and neck cancer. A cancer can also be a brain cancer or another type of CNS or intracranial tumor. For example, a subject can have an astrocytic tumor (e.g., astrocytoma, anaplastic astrocytoma, glioblastoma, pilocytic astrocytoma, subependymal giant cell astrocytoma, pleomorphic xanthoastrocytoma), oligodendroglial tumor (e.g., oligodendroglioma, anaplastic oligodendroglioma), ependymal cell tumor (e.g., ependymoma, anaplastic ependymoma, myxopapillary ependymoma, subependymoma), mixed glioma (e.g., mixed oligoastrocytoma, anaplastic oligoastrocytoma), neuroepithelial tumor of uncertain origin (e.g., polar spongioblastoma, astroblastoma, gliomatosis cerebri), tumor of the choroid plexus (e.g., choroid plexus papilloma, choroid plexus carcinoma), neuronal or mixed neuronal-glial tumor (e.g., gangliocytoma, dyplastic gangliocytoma of cerebellum, ganglioglioma, anaplastic ganglioglioma, desmoplastic infantile ganglioma, central neurocytoma, dysembryoplastic neuroepthelial tumor, olfactory neuroblastoma), pineal parenchyma tumor (e.g., pineocytoma, pineoblastoma, mixed pineocytoma/pineoblastoma), or tumor with mixed neuroblastic or glioblastic elements (e.g., medulloepithelioma, medulloblastoma, neuroblastoma, retinoblastoma, ependymoblastoma).

[00325] The term “treat” or “treating” refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or lessen the symptoms of the targeted disease. Treating may include one or more of directly affecting or curing, suppressing, inhibiting, preventing, reducing the severity of, delaying the onset of, slowing the progression of, stabilizing the progression of, inducing remission of, preventing or delaying the metastasis of, reducing/ameliorating symptoms associated with the disease, or a combination thereof. For example, treating may include increasing expected survival time. The effect (e.g., suppressing, inhibiting, preventing, reducing the severity of, delaying the onset of, slowing the progression of, stabilizing the progression of, inducing remission of, preventing or delaying, reducing/ameliorating symptoms of, and so forth, can be relative to a control subject not

receiving a treatment or receiving a placebo treatment. The term "treat" or "treating" can also refer to increasing percent chance of survival or increasing expected time of survival for a subject with the disease (e.g., relative to a control subject not receiving a treatment or receiving a placebo treatment). In one example, "treating" refers to delaying progression, expediting remission, inducing remission, augmenting remission, speeding recovery, increasing efficacy of alternative therapeutics, decreasing resistance to alternative therapeutics, or a combination thereof (e.g., relative to a control subject not receiving a treatment or receiving a placebo treatment). The terms "preventing" or "impeding" can refer, for example to delaying the onset of symptoms, preventing relapse of a disease, decreasing the number or frequency of relapse episodes, increasing latency between symptomatic episodes, or a combination thereof. The terms "suppressing" or "inhibiting" can refer, for example, to reducing the severity of symptoms, reducing the severity of an acute episode, reducing the number of symptoms, reducing the incidence of disease-related symptoms, reducing the latency of symptoms, ameliorating symptoms, reducing secondary symptoms, reducing secondary infections, prolonging patient survival, or a combination thereof.

[00326] The term "subject" refers to a mammal (e.g., a human) in need of therapy for, or susceptible to developing, a disease. The term subject also refers to a mammal (e.g., a human) that receives either prophylactic or therapeutic treatment. The subject may include dogs, cats, pigs, cows, sheep, goats, horses, rats, mice, non-human mammals, and humans. The term "subject" does not necessarily exclude an individual that is healthy in all respects and does not have or show signs of the disease.

[00327] An individual is at increased risk of developing a disease if the subject has at least one known risk-factor (e.g., genetic, biochemical, family history, and situational exposure) placing individuals with that risk factor at a statistically significant greater risk of developing the disease than individuals without the risk factor.

[00328] A "symptom" or "sign" refers to objective evidence of a disease as observed by a physician or subjective evidence of a disease, such as altered gait, as perceived by the subject. A symptom or sign may be any manifestation of a disease. Symptoms can be primary or secondary. The term "primary" refers to a symptom that is a direct result of a particular disease or disorder (e.g., a tumor or cancer), while the term "secondary" refers to a symptom that is derived from or consequent to a primary cause. The lyophilized or reconstituted recombinant

bacteria or *Listeria* strains, the immunogenic compositions, the pharmaceutical compositions, and the vaccines disclosed herein can treat primary or secondary symptoms or secondary complications.

[00329] The lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines are administered in an effective regime, meaning a dosage, route of administration, and frequency of administration that delays the onset, reduces the severity, inhibits further deterioration, and/or ameliorates at least one sign or symptom of the disease. Alternatively, the lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines are administered in an effective regime, meaning a dosage, route of administration, and frequency of administration that induces an immune response to a disease-associated antigen in the lyophilized or reconstituted recombinant bacteria or *Listeria* strain, the immunogenic composition, the pharmaceutical composition, or the vaccine, or that induces an immune response to the bacteria or *Listeria* strain itself. If a subject is already suffering from the disease, the regime can be referred to as a therapeutically effective regime. If the subject is at elevated risk of developing the disease relative to the general population but is not yet experiencing symptoms, the regime can be referred to as a prophylactically effective regime. In some instances, therapeutic or prophylactic efficacy can be observed in an individual patient relative to historical controls or past experience in the same patient. In other instances, therapeutic or prophylactic efficacy can be demonstrated in a preclinical or clinical trial in a population of treated patients relative to a control population of untreated patients. For example, a regime can be considered therapeutically or prophylactically effective if an individual treated patient achieves an outcome more favorable than the mean outcome in a control population of comparable patients not treated by methods described herein, or if a more favorable outcome is demonstrated in treated patients versus control patients in a controlled clinical trial (e.g., a phase II, phase II/III or phase III trial) at the $p < 0.05$ or 0.01 or even 0.001 level.

[00330] Exemplary dosages for a recombinant *Listeria* strain are, for example, 1×10^6 - 1×10^7 CFU, 1×10^7 - 1×10^8 CFU, 1×10^8 - 3.31×10^{10} CFU, 1×10^9 - 3.31×10^{10} CFU, 5-500×10^8 CFU, 7-500×10^8 CFU, 10-500×10^8 CFU, 20-500×10^8 CFU, 30-500×10^8 CFU, 50-500×10^8 CFU, 70-500×10^8 CFU, 100-500×10^8 CFU, 150-500×10^8 CFU, 5-300×10^8 CFU, 5-200×10^8 CFU, 5-15×10^8 CFU, 5-100×10^8 CFU, 5-70×10^8 CFU, 5-50×10^8 CFU, 5-30×10^8 CFU, 5-20×10^8 CFU,

1-30×10^9 CFU, 1-20×10^9CFU, 2-30×10^9 CFU, 1-10×10^9 CFU, 2-10×10^9 CFU, 3-10×10^9 CFU, 2-7×10^9 CFU, 2-5×10^9 CFU, and 3-5×10^9 CFU. Other exemplary dosages for a recombinant *Listeria* strain are, for example, 1×10^7 organisms, 1.5×10^7 organisms, 2×10^8 organisms, 3×10^7 organisms, 4×10^7 organisms, 5×10^7 organisms, 6×10^7 organisms, 7×10^7 organisms, 8×10^7 organisms, 10×10^7 organisms, 1.5×10^8 organisms, 2×10^8 organisms, 2.5×10^8 organisms, 3×10^8 organisms, 3.3×10^8 organisms, 4×10^8 organisms, 5×10^8 organisms, 1×10^9 organisms, 1.5×10^9 organisms, 2×10^9 organisms, 3×10^9 organisms, 4×10^9 organisms, 5×10^9 organisms, 6×10^9 organisms, 7×10^9 organisms, 8×10^9 organisms, 10×10^9 organisms, 1.5×10^{10} organisms, 2×10^{10} organisms, 2.5×10^{10} organisms, 3×10^{10} organisms, 3.3×10^{10} organisms, 4×10^{10} organisms, and 5×10^{10} organisms. The dosage can depend on the condition of the patient and response to prior treatment, if any, whether the treatment is prophylactic or therapeutic, and other factors.

[00331] Administration can be by any suitable means. For example, administration can be parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intracerebroventricular, intraperitoneal, topical, intranasal, intramuscular, intra-ocular, intrarectal, conjunctival, transdermal, intradermal, vaginal, rectal, intratumoral, parcanceral, transmucosal, intravascular, intraventricular, inhalation (aerosol), nasal aspiration (spray), sublingual, aerosol, suppository, or a combination thereof. For intranasal administration or application by inhalation, solutions or suspensions of the recombinant fusion polypeptides, nucleic acids encoding recombinant fusion polypeptides, recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines mixed and aerosolized or nebulized in the presence of the appropriate carrier are suitable. Such an aerosol may comprise any lyophilized or reconstituted recombinant bacteria or *Listeria* strain, immunogenic composition, pharmaceutical composition, or vaccine described herein. Administration may also be in the form of a suppository (e.g., rectal suppository or urethral suppository), in the form of a pellet for subcutaneous implantation (e.g., providing for controlled release over a period of time), or in the form of a capsule. Administration may also be via injection into a disease site. Regimens of administration can be readily determined based on factors such as exact nature and type of the disease being treated, the severity of the disease, the age and general physical condition of the subject, body weight of the subject, response of the individual subject, and the like.

[00332] The frequency of administration can depend on the half-life of the lyophilized or

reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines in the subject, the condition of the subject, and the route of administration, among other factors. The frequency can be, for example, daily, weekly, monthly, quarterly, or at irregular intervals in response to changes in the subject's condition or progression of the tumor or cancer being treated. The course of treatment can depend on the condition of the subject and other factors. For example, the course of treatment can be several weeks, several months, or several years (e.g., up to 2 years). For example, repeat administrations (doses) may be undertaken immediately following the first course of treatment or after an interval of days, weeks or months to achieve disease regression or suppression. Assessment may be determined by any known technique, including diagnostic methods such as imaging techniques, analysis of serum biomarkers, biopsy, or the presence, absence, or amelioration of disease-associated symptoms. As a specific example, the lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines can be administered every 3 weeks for up to 2 years. In one example, a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein is administered in increasing doses in order to increase the T-effector cell to regulatory T cell ratio and generate a more potent anti-disease immune response. Anti-disease immune responses can be further strengthened by providing the subject with cytokines including, for example, IFN-γ, TNF-α, and other cytokines known to enhance cellular immune response. *See, e.g.,* US 6,991,785, herein incorporated by reference in its entirety for all purposes.

[00333] Some methods may further comprise "boosting" the subject with additional lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines or administering the lyophilized or reconstituted recombinant bacteria or *Listeria* strains, immunogenic compositions, pharmaceutical compositions, or vaccines multiple times. "Boosting" refers to administering an additional dose to a subject. For example, in some methods, 2 boosts (or a total of 3 inoculations) are administered, 3 boosts are administered, 4 boosts are administered, 5 boosts are administered, or 6 or more boosts are administered. The number of dosages administered can depend on, for example, the response of the disease to the treatment.

[00334] Optionally, the lyophilized or reconstituted recombinant bacteria or *Listeria* strain,

immunogenic composition, pharmaceutical composition, or vaccine used in the booster inoculation is the same as the lyophilized or reconstituted recombinant bacteria or *Listeria* strain, immunogenic composition, pharmaceutical composition, or vaccine used in the initial "priming" inoculation. Alternatively, the booster is different from the priming recombinant bacteria or *Listeria* strain, immunogenic composition, pharmaceutical composition, or vaccine. Optionally, the same dosages are used in the priming and boosting inoculations. Alternatively, a larger dosage is used in the booster, or a smaller dosage is used in the booster. The period between priming and boosting inoculations can be experimentally determined. For example, the period between priming and boosting inoculations can be 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6-8 weeks, or 8-10 weeks.

[00335] Heterologous prime boost strategies have been effective for enhancing immune responses and protection against numerous pathogens. *See, e.g.*, Schneider et al. (1999) *Immunol. Rev.* 170:29-38; Robinson (2002) *Nat. Rev. Immunol.* 2:239-250; Gonzalo et al. (2002) *Vaccine* 20:1226-1231; and Tanghe (2001) *Infect. Immun.* 69:3041-3047, each of which is herein incorporated by reference in its entirety for all purposes. Providing antigen in different forms in the prime and the boost injections can maximize the immune response to the antigen. DNA vaccine priming followed by boosting with protein in adjuvant or by viral vector delivery of DNA encoding antigen is one effective way of improving antigen-specific antibody and $CD4^+$ T-cell responses or $CD8^+$ T-cell responses. *See, e.g.*, Shiver et al. (2002) *Nature* 415: 331-335; Gilbert et al. (2002) *Vaccine* 20:1039-1045; Billaut-Mulot et al. (2000) *Vaccine* 19:95-102; and Sin et al. (1999) *DNA Cell Biol.* 18:771-779, each of which is herein incorporated by reference in its entirety for all purposes. As one example, adding CRL1005 poloxamer (12 kDa, 5% POE) to DNA encoding an antigen can enhance T-cell responses when subjects are vaccinated with a DNA prime followed by a boost with an adenoviral vector expressing the antigen. *See, e.g.*, Shiver et al. (2002) *Nature* 415:331-335, herein incorporated by reference in its entirety for all purposes. As another example, a vector construct encoding an immunogenic portion of an antigen and a protein comprising the immunogenic portion of the antigen can be administered. *See, e.g.,* US 2002/0165172, herein incorporated by reference in its entirety for all purposes. Similarly, an immune response of nucleic acid vaccination can be enhanced by simultaneous administration of (e.g., during the same immune response, in some embodiments within 0-10 or 3-7 days of each other) a polynucleotide and polypeptide of interest. *See, e.g.*, US 6,500,432,

herein incorporated by reference in its entirety for all purposes.

[00336] The therapeutic methods disclosed herein can also comprise administering one or more additional compounds effective in preventing or treating a disease (e.g., a tumor or cancer). For example, an additional compound may comprise a compound useful in chemotherapy, such as amsacrine, bleomycin, busulfan, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clofarabine, crisantaspase, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, fludarabine, fluorouracil (5-FU), gemcitabine, gliadelimplants, hydroxycarbamide, idarubicin, ifosfamide, irinotecan, leucovorin, liposomaldoxorubicin, liposomaldaunorubicin, lomustine, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitoxantrone, oxaliplatin, paclitaxel (Taxol), pemetrexed, pentostatin, procarbazine, raltitrexed, satraplatin, streptozocin, tegafur-uracil, temozolomide, teniposide, thiotepa, tioguanine, topotecan, treosulfan, vinblastine, vincristine, vindesine, vinorelbine, or a combination thereof. Alternatively, an additional compound can also comprise other biologics, including Herceptin® (trastuzumab) against the HER2 antigen, Avastin® (bevacizumab) against VEGF, or antibodies to the EGF receptor, such as Erbitux® (cetuximab), and Vectibix® (panitumumab). Alternatively, an additional compound can comprise other immunotherapies. Alternatively, the additional compound can be an indoleamine 2,3-dioxygenase (IDO) pathway inhibitor, such as 1-methyltryptophan (1MT), 1-methyltryptophan (1MT), Necrostatin-1, Pyridoxal Isonicotinoyl Hydrazone, Ebselen, 5-Methylindole-3-carboxaldehyde, CAY10581, an anti-IDO antibody, or a small molecule IDO inhibitor. IDO inhibition can enhance the efficacy of chemotherapeutic agents. The therapeutic methods disclosed herein can also be combined with radiation, stem cell treatment, surgery, or any other treatment.

[00337] Such additional compounds or treatments can precede the administration of a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein, follow the administration of a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein, or be simultaneous to the administration of a lyophilized or reconstituted recombinant bacteria or *Listeria* strain, an immunogenic composition, a pharmaceutical composition, or a vaccine disclosed herein.

[00338] Targeted immunomodulatory therapy is focused primarily on the activation of costimulatory receptors, for example by using agonist antibodies that target members of the tumor necrosis factor receptor superfamily, including 4-1BB, OX40, and GITR (glucocorticoid-induced TNF receptor-related). The modulation of GITR has demonstrated potential in both antitumor and vaccine settings. Another target for agonist antibodies are co-stimulatory signal molecules for T cell activation. Targeting costimulatory signal molecules may lead to enhanced activation of T cells and facilitation of a more potent immune response. Co-stimulation may also help prevent inhibitory influences from checkpoint inhibition and increase antigen-specific T cell proliferation.

[00339] *Listeria*-based immunotherapy acts by inducing the *de novo* generation of tumor antigen-specific T cells that infiltrate and destroy the tumor and by reducing the numbers and activities of immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment. Antibodies (or functional fragments thereof) for T cell co-inhibitory or co-stimulatory receptors (e.g., checkpoint inhibitors CTLA-4, PD-1, TIM-3, LAG3 and co-stimulators CD137, OX40, GITR, and CD40) can have synergy with *Listeria*-based immunotherapy.

[00340] Thus, some methods can comprise further administering a composition comprising an immune checkpoint inhibitor antagonist, such as a PD-1 signaling pathway inhibitor, a CD-80/86 and CTLA-4 signaling pathway inhibitor, a T cell membrane protein 3 (TIM3) signaling pathway inhibitor, an adenosine A2a receptor (A2aR) signaling pathway inhibitor, a lymphocyte activation gene 3 (LAG3) signaling pathway inhibitor, a killer immunoglobulin receptor (KIR) signaling pathway inhibitor, a CD40 signaling pathway inhibitor, or any other antigen-presenting cell/T cell signaling pathway inhibitor. Examples of immune checkpoint inhibitor antagonists include an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof. For example, an anti PD-1 antibody can be administered to a subject at 5-10 mg/kg every 2 weeks, 5-10 mg/kg every 3 weeks, 1-2 mg/kg every 3 weeks, 1-10 mg/kg every week, 1-10 mg/kg every 2 weeks, 1-10 mg/kg every 3 weeks, or 1-10 mg/kg every 4 weeks.

[00341] Likewise, some methods can further comprise administering a T cell stimulator, such as an antibody or functional fragment thereof binding to a T-cell receptor co-stimulatory molecule, an antigen presenting cell receptor binding co-stimulatory molecule, or a member of

the TNF receptor superfamily. The T-cell receptor co-stimulatory molecule can comprise, for example, CD28 or ICOS. The antigen presenting cell receptor binding co-stimulatory molecule can comprise, for example, a CD80 receptor, a CD86 receptor, or a CD46 receptor. The TNF receptor superfamily member can comprise, for example, glucocorticoid-induced TNF receptor (GITR), OX40 (CD134 receptor), 4-1BB (CD137 receptor), or TNFR25.

[00342] For example, some methods can further comprise administering an effective amount of a composition comprising an antibody or functional fragment thereof binding to a T-cell receptor co-stimulatory molecule or an antibody or functional fragment thereof binding to an antigen presenting cell receptor binding a co-stimulatory molecule. The antibody can be, for example, an anti-TNF receptor antibody or antigen-binding fragment thereof (e.g., TNF receptor superfamily member glucocorticoid-induced TNF receptor (GITR), OX40 (CD134 receptor), 4-1BB (CD137 receptor), or TNFR25), an anti-OX40 antibody or antigen-binding fragment thereof, or an anti-GITR antibody or antigen binding fragment thereof. Alternatively, other agonistic molecules can be administered (e.g., GITRL, an active fragment of GITRL, a fusion protein containing GITRL, a fusion protein containing an active fragment of GITRL, an antigen presenting cell (APC)/T cell agonist, CD134 or a ligand or fragment thereof, CD137 or a ligand or fragment thereof, or an inducible T cell costimulatory (ICOS) or a ligand or fragment thereof, or an agonistic small molecule).

[00343] In a specific example, some methods can further comprise administering an anti-CTLA-4 antibody or a functional fragment thereof and/or an anti-CD137 antibody or functional fragment thereof. For example, the anti-CTLA-4 antibody or a functional fragment thereof or the anti-CD137 antibody or functional fragment thereof can be administered about 72 hours after the first dose of recombinant fusion polypeptide, nucleic acids encoding a recombinant fusion polypeptide, recombinant bacteria or *Listeria* strain, immunogenic composition, pharmaceutical composition, or vaccine, or about 48 hours after the first dose of recombinant fusion polypeptide, nucleic acids encoding a recombinant fusion polypeptide, recombinant bacteria or *Listeria* strain, immunogenic composition, pharmaceutical composition, or vaccine. The anti-CTLA-4 antibody or a functional fragment thereof or anti-CD137 antibody or functional fragment thereof can be administered at a dose, for example, of about 0.05 mg/kg and about 5 mg/kg. A recombinant *Listeria* strain or immunogenic composition comprising a recombinant *Listeria* strain can be administered at a dose, for example, of about 1×10^9 CFU. Some such methods can further

comprise administering an effective amount of an anti-PD-1 antibody or functional fragment thereof.

[00344] Methods for assessing efficacy of cancer immunotherapies are well-known and are described, for example, in Dzojic et al. (2006) *Prostate* 66(8):831-838; Naruishi et al. (2006) *Cancer Gene Ther.* 13(7):658-663, Sehgal et al. (2006) *Cancer Cell Int.* 6:21), and Heinrich et al. (2007) *Cancer Immunol Immunother* 56(5):725-730, each of which is herein incorporated by reference in its entirety for all purposes. As one example, for prostate cancer, a prostate cancer model can be to test methods and compositions disclosed herein, such as a TRAMP-C2 mouse model, a 178-2 BMA cell model, a PAIII adenocarcinoma cells model, a PC-3M model, or any other prostate cancer model.

[00345] Alternatively or additionally, the immunotherapy can be tested in human subjects, and efficacy can be monitored using known. Such methods can include, for example, directly measuring CD4+ and CD8+ T cell responses, or measuring disease progression (e.g., by determining the number or size of tumor metastases, or monitoring disease symptoms such as cough, chest pain, weight loss, and so forth). Methods for assessing the efficacy of a cancer immunotherapy in human subjects are well-known and are described, for example, in Uenaka et al. (2007) *Cancer Immun.* 7:9 and Thomas-Kaskel et al. (2006) *Int J Cancer* 119(10):2428-2434, each of which is herein incorporated by reference in its entirety for all purposes.

VII. Kits

[00346] Also provided are kits comprising a reagent utilized in performing a method disclosed herein or kits comprising a composition, tool, or instrument disclosed herein.

[00347] For example, such kits can comprise a lyophilized recombinant bacteria or *Listeria* strain disclosed herein, an immunogenic composition disclosed herein, a pharmaceutical composition disclosed herein, or a vaccine disclosed herein. Such kits can also comprise a solvent or diluent for reconstituting the lyophilized recombinant bacteria or *Listeria* strain. In addition, such kits can additionally comprise an instructional material which describes use of the lyophilized recombinant bacteria or *Listeria* strain, the immunogenic composition, the pharmaceutical composition, or the vaccine to perform the methods disclosed herein. Such kits can optionally further comprise an applicator. Although model kits are described below, the contents of other useful kits will be apparent in light of the present disclosure.

[00348] All patent filings, websites, other publications, accession numbers and the like cited above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the invention can be used in combination with any other unless specifically indicated otherwise. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

LISTING OF EMBODIMENTS

[00349] The subject matter disclosed herein includes, but is not limited to, the following embodiments.

[00350] 1. A method for producing a lyophilized composition comprising a *Listeria* strain, comprising: (a) providing a composition comprising a *Listeria* strain in a formulation comprising a buffer and sucrose; (b) cooling the composition provided in step (a) at a holding temperature between about −32°C and about −80°C in a freezing step; (c) exposing the composition produced by step (b) to a vacuum at a holding temperature between about −10°C and about −30°C in a primary drying step; and (d) exposing the composition produced by step (c) to a vacuum at a holding temperature between about −5°C and about 25°C in a secondary drying step, whereby the lyophilized composition is produced.

[00351] 2. The method of embodiment 1, wherein prior to step (a), a stress response is induced in the *Listeria* strain by exposing the *Listeria* strain to a decreased temperature.

[00352] 3. The method of embodiment 1, wherein prior to step (a), a stress response is not induced in the *Listeria* strain by exposing the *Listeria* strain to a decreased temperature.

[00353] 4. The method of any preceding embodiment, wherein the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that is thawed prior to step (a).

[00354] 5. The method of embodiment 4, wherein the concentration of the frozen *Listeria* strain being thawed is between about 1x10E9 to about 1x10E10 colony forming units (CFU) per milliliter.

[00355] 6. The method of embodiment 4 or 5, wherein the frozen *Listeria* strain is thawed at about 2°C to about 37°C.

[00356] 7. The method of embodiment 6, wherein the frozen *Listeria* strain is thawed at about 20°C to about 37°C.

[00357] 8. The method of embodiment 7, wherein the frozen *Listeria* strain is thawed at about 32°C and about 37°C.

[00358] 9. The method of embodiment 8, wherein the frozen *Listeria* strain is thawed at about 37°C.

[00359] 10. The method of any one of embodiments 4-9, wherein the frozen *Listeria* strain is thawed for no more than 8 hours.

[00360] 11. The method of any one of embodiments 4-10, wherein the frozen *Listeria* strain is held at about 2°C to about 8°C for no more than 24 hours after thawing.

[00361] 12. The method of any one of embodiments 1-3, wherein the *Listeria* strain used in the composition in step (a) is freshly cultured prior to step (a).

[00362] 13. The method of any preceding embodiment, wherein the buffer is a phosphate buffer.

[00363] 14. The method of any preceding embodiment, wherein the formulation comprises about 1% to about 5% w/v sucrose.

[00364] 15. The method of embodiment 14, wherein the formulation comprises about 2% to about 3% w/v sucrose.

[00365] 16. The method of embodiment 15, wherein the formulation comprises about 2.5% w/v sucrose.

[00366] 17. The method of any preceding embodiment, wherein the formulation comprises about 1x10E9 to about 1x10E10 colony forming units (CFU) of *Listeria* per milliliter.

[00367] 18. The method of any preceding embodiment, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00368] 19. The method of embodiment 18, wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00369] 20. The method of any preceding embodiment, wherein the holding temperature in the freezing step (b) is between about −40°C and about −50°C.

[00370] 21. The method of embodiment 20, wherein the holding temperature in the freezing step (b) is about −45°C.

[00371] 22. The method of any preceding embodiment, wherein the freezing step (b) comprises decreasing the temperature to the holding temperature at a rate of about 1°C per minute.

[00372] 23. The method of any preceding embodiment, wherein the cooling in the freezing step (b) is for about 2 hours to about 4 hours.

[00373] 24. The method any preceding embodiment, wherein cooling in the freezing step (b) comprises holding the composition at the holding temperature for about 2 hours.

[00374] 25. The method of any preceding embodiment, wherein the holding temperature in the primary drying step (c) is between about −12°C and about −22°C.

[00375] 26. The method of embodiment 25, wherein the holding temperature in the primary drying step (c) is between about −17°C and about −19°C.

[00376] 27. The method of embodiment 26, wherein the holding temperature in the primary drying step (c) is about −18°C.

[00377] 28. The method of any preceding embodiment, wherein the primary drying step (c) comprises increasing the temperature to the holding temperature at a rate of about 1°C per minute.

[00378] 29. The method of any preceding embodiment, wherein the primary drying step (c) is for about 25 hours to about 35 hours.

[00379] 30. The method of any preceding embodiment, wherein the end of the primary drying step (c) is about 12 to about 16 hours after the composition has reached holding temperature.

[00380] 31. The method of any preceding embodiment, wherein the primary drying step (c) is at a vacuum pressure of about 0.09 mbar.

[00381] 32. The method of any preceding embodiment, wherein the holding temperature in the secondary drying step (d) is between about −5°C and about 20°C.

[00382] 33. The method of embodiment 32, wherein the holding temperature in the secondary drying step (d) is between about −5°C and about 5°C.

[00383] 34. The method of embodiment 33, wherein the holding temperature in the secondary drying step (d) is about 0°C.

[00384] 35. The method of any preceding embodiment, wherein the secondary drying step (d) comprises increasing the temperature to the holding temperature at a rate of about 0.2°C per minute.

[00385] 36. The method of any preceding embodiment, wherein the secondary drying step (d) is for about 1 hour to about 10 hours.

[00386] 37. The method any preceding embodiment, wherein the secondary drying step (d) comprises holding the composition at the holding temperature for about 2 hours to about 6 hours.

[00387] 38. The method any embodiment 37, wherein the secondary drying step (d) comprises holding the composition at the holding temperature for about 5 hours to about 6 hours.

[00388] 39. The method of any preceding embodiment, wherein the secondary drying step (d) is at a vacuum pressure of about 0.09 mbar.

[00389] 40. The method of any preceding embodiment, wherein the residual moisture in the lyophilized composition is between about 1% and about 5%.

[00390] 41. The method of embodiment 40, wherein the residual moisture in the lyophilized composition is between about 2% and about 4%.

[00391] 42. The method of any preceding embodiment, wherein the residual moisture in the lyophilized composition is at least about 2.5%.

[00392] 43. The method of embodiment 42, wherein the residual moisture in the lyophilized composition is at least about 3%.

[00393] 44. The method of any preceding embodiment, wherein the lyophilized composition shows at least about 60% viability after storage at between about −20°C and about 4°C for about 12 months.

[00394] 45. The method of embodiment 44, wherein the lyophilized composition shows at least about 75% viability after storage at between about −20°C and about 4°C for about 12 months.

[00395] 46. The method of embodiment 45, wherein the lyophilized composition shows at least about 80% viability after storage at between about –20°C and about 4°C for about 12 months.

[00396] 47. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00397] 48. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the buffer is a phosphate buffer, and wherein the formulation comprises about 2% to about 3% w/v sucrose, and wherein the formulation does not comprise trehalose, MSG, or rHSA, and wherein the formulation comprises about 1x10E9 to about 1x10E10 colony forming units (CFU) of *Listeria* per milliliter, and wherein the holding temperature in the freezing step (a) is between about –40°C and about –50°C, and wherein the holding temperature in the primary drying step (c) is between –17°C and –19°C, and wherein the holding temperature in the secondary drying step (d) is between –1°C and 1°C, and wherein the residual moisture in the lyophilized composition is between about 2.5% and about 4%.

[00398] 49. The method of 48, wherein the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that is thawed prior to step (a), and wherein the concentration of the frozen *Listeria* strain being thawed is between about 1x10E9 to about 1x10E10 colony forming units (CFU) per milliliter, and wherein the frozen *Listeria* strain is thawed at about 37°C, and wherein the frozen *Listeria* strain is thawed for no more than 8 hours, and wherein the frozen *Listeria* strain is held at about 2°C to about 8°C for no more than 24 hours after thawing.

[00399] 50. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00400] 51. The method of embodiment 50, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00401] 52. The method of embodiment 50, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in

actA, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

[00402] 53. A formulation for lyophilization of a *Listeria* strain, comprising: (1) the *Listeria* strain; (2) a phosphate buffer; and (3) sucrose.

[00403] 54. The formulation of embodiment 53, wherein the *Listeria* strain is a strain in which a stress response has been induced by exposing the *Listeria* strain to a decreased temperature.

[00404] 55. The formulation of embodiment 53 or 54, wherein the *Listeria* strain is from a frozen *Listeria* stock.

[00405] 56. The formulation of embodiment 53 or 54, wherein the *Listeria* strain is from a freshly cultured *Listeria* stock.

[00406] 57. The formulation of any one of embodiments 53-56, wherein the formulation comprises about 1% to about 5% w/v sucrose.

[00407] 58. The formulation of embodiment 57, wherein the formulation comprises about 2% to about 3% w/v sucrose.

[00408] 59. The formulation of embodiment 58, wherein the formulation comprises about 2.5% w/v sucrose.

[00409] 60. The formulation of any one of embodiments 53-59, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00410] 61. The formulation of embodiment 60, wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00411] 62. The formulation of any one of embodiments 53-61, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00412] 63. The formulation of any one of embodiments 53-62, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the formulation comprises about 2% to about 3% w/v sucrose, and wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00413] 64. The formulation of any one of embodiments 53-63, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00414] 65. The formulation of embodiment 64, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00415] 66. The formulation of embodiment 64, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

[00416] 67. A lyophilized composition produced by the method of any one of embodiments 1-52.

[00417] 68. A lyophilized composition comprising a *Listeria* strain, a phosphate buffer, and sucrose.

[00418] 69. The lyophilized composition of embodiment 68, wherein the lyophilized composition does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00419] 70. The lyophilized composition of embodiment 69, wherein the lyophilized composition does not comprise trehalose, MSG, or rHSA.

[00420] 71. The lyophilized composition of any one of embodiments 67-70, wherein the residual moisture in the lyophilized composition is between about 1% and about 5%.

[00421] 72. The lyophilized composition of embodiment 71, wherein the residual moisture in the lyophilized composition is between about 2% and about 4%.

[00422] 73. The lyophilized composition of any one of embodiments 67-72, wherein the residual moisture in the lyophilized composition is at least about 2.5%.

[00423] 74. The lyophilized composition of embodiment 73, wherein the residual moisture in the lyophilized composition is at least about 3%.

[00424] 75. A lyophilized composition comprising a *Listeria* strain, wherein the residual moisture in the lyophilized composition is at least about 2.5%.

[00425] 76. The lyophilized composition of any one of embodiments 67-75, wherein the lyophilized composition shows at least about 60% viability after storage at between about –20°C and about 4°C for about 12 months.

[00426] 77. The lyophilized composition of embodiment 76, wherein the lyophilized composition shows at least about 75% viability after storage at between about –20°C and about 4°C for about 12 months.

[00427] 78. The lyophilized composition of embodiment 77, wherein the lyophilized composition shows at least about 80% viability after storage at between about –20°C and about 4°C for about 12 months.

[00428] 79. The lyophilized composition of any one of embodiments 67-78, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00429] 80. The lyophilized composition of any one of embodiments 67-79, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the lyophilized composition does not comprise trehalose, MSG, or rHSA, and wherein the residual moisture in the lyophilized composition is between 2.5% and 4%.

[00430] 81. The lyophilized composition of any one of embodiments 67-80, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00431] 82. The lyophilized composition of embodiment 81, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00432] 83. The lyophilized composition of embodiment 81, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

[00433] 84. A method of preparing a frozen *Listeria* strain for lyophilization, comprising thawing the frozen *Listeria* strain at a temperature between about 20°C and about 37°C.

[00434] 85. The method of embodiment 84, wherein the temperature is between about 32°C and about 37°C.

[00435] 86. The method of embodiment 85, wherein the temperature is about 37°C.

[00436] 87. The method of any one of embodiments 84-86, wherein the frozen *Listeria* strain is thawed for no more than 8 hours.

[00437] 88. The method of any one of embodiments 84-87, wherein the frozen *Listeria* strain is held at about 2°C to about 8°C for no more than 24 hours after thawing.

[00438] 89. The method of any one of embodiments 84-88, wherein the frozen *Listeria* strain is thawed in a formulation comprising a buffer and sucrose.

[00439] 90. The method of embodiment 89, wherein the formulation comprises about 1% to about 5% w/v sucrose.

[00440] 91. The method of embodiment 90, wherein the formulation comprises about 2% to about 3% w/v sucrose.

[00441] 92. The method of embodiment 91, wherein the formulation comprises about 2.5% w/v sucrose.

[00442] 93. The method of any one of embodiments 89-92, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00443] 94. The method of embodiment 93, wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00444] 95. The method of any one of embodiments 89-94, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00445] 96. The method of any one of embodiments 89-95, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the formulation comprises about 2% to about 3% w/v sucrose, and wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00446] The subject matter disclosed herein also includes, but is not limited to, the following embodiments.

[00447] 1. A method for producing a lyophilized composition comprising a *Listeria* strain, comprising: (a) providing a composition comprising a *Listeria* strain in a formulation comprising a buffer and sucrose; (b) cooling the composition provided in step (a) in a freezing step, optionally wherein the temperature is between about −32°C and −80°C; (c) exposing the composition produced by step (b) to a vacuum in a primary drying step, optionally wherein the temperature is between about −10°C and −30°C; and (d) exposing the composition produced by step (c) to a vacuum in a secondary drying step, optionally wherein the temperature is between

about 5°C and 25°C, optionally wherein the temperature is between about 5°C and 20°C, whereby the lyophilized composition is produced.

[00448] 2. The method of embodiment 1, wherein prior to step (a), a stress response is induced in the *Listeria* strain by exposing the *Listeria* strain to a decreased temperature.

[00449] 3. The method of embodiment 1, wherein prior to step (a), a stress response is not induced in the *Listeria* strain by exposing the *Listeria* strain to a decreased temperature.

[00450] 4. The method of any preceding embodiment, wherein the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that was thawed prior to step (a).

[00451] 5. The method of any one of embodiments 1-3, wherein the *Listeria* strain used in the composition in step (a) was freshly cultured prior to step (a).

[00452] 6. The method of any preceding embodiment, wherein the buffer is a phosphate buffer.

[00453] 7. The method of any preceding embodiment, wherein the formulation comprises 1% to 5% w/v sucrose.

[00454] 8. The method of embodiment 7, wherein the formulation comprises 2% to 3% w/v sucrose.

[00455] 9. The method of any preceding embodiment, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00456] 10. The method of embodiment 9, wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00457] 11. The method of any preceding embodiment, wherein the temperature in the freezing step (b) is between −40°C and −50°C.

[00458] 12. The method of any preceding embodiment, wherein the cooling in the freezing step (b) is for 2-4 hours.

[00459] 13. The method of any preceding embodiment, wherein the temperature in the primary drying step (c) is between −12°C and −22°C.

[00460] 14. The method of embodiment 13, wherein the temperature in the primary drying step (c) is between −17°C and −19°C.

[00461] 15. The method of any preceding embodiment, wherein the primary drying the primary drying step (c) is for 20-30 hours.

[00462] 16. The method of any preceding embodiment, wherein the temperature in the secondary drying step (d) is between 10°C and 20°C.

[00463] 17. The method of any preceding embodiment, wherein the secondary drying step (d) is for 1-10 hours.

[00464] 18. The method of embodiment 17, wherein the secondary drying step (d) is for 1-3 hours.

[00465] 19. The method of any preceding embodiment, wherein the residual moisture in the lyophilized composition is between 1% and 5%.

[00466] 20. The method of embodiment 19, wherein the residual moisture in the lyophilized composition is between 3% and 4%.

[00467] 21. The method of any preceding embodiment, wherein the lyophilized composition shows at least 60% viability after storage at –20°C or 4°C for 6 months.

[00468] 22. The method of embodiment 21, wherein the lyophilized composition shows at least 75% viability after storage at –20°C or 4°C for 6 months.

[00469] 23. The method of embodiment 22, wherein the lyophilized composition shows at least 80% viability after storage at –20°C or 4°C for 6 months.

[00470] 24. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00471] 25. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the buffer is a phosphate buffer, and wherein the formulation comprises 2% to 3% w/v sucrose, and wherein the formulation does not comprise trehalose, MSG, or rHSA, and wherein the temperature in the primary drying step (c) is between –17°C and –19°C, wherein the temperature in the secondary drying step (d) is between 10°C and 20°C, and wherein the residual moisture in the lyophilized composition is between 3% and 4%.

[00472] 26. The method of any preceding embodiment, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00473] 27. The method of embodiment 26, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in

prfA, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00474] 28. The method of embodiment 26, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

[00475] 29. A formulation for lyophilization of a *Listeria* strain, comprising: (1) the *Listeria* strain; (2) a phosphate buffer; and (3) sucrose.

[00476] 30. The formulation of embodiment 29, wherein the *Listeria* strain is a strain in which a stress response has been induced by exposing the *Listeria* strain to a decreased temperature.

[00477] 31. The formulation of embodiment 29 or 30, wherein the *Listeria* strain is from a frozen *Listeria* stock.

[00478] 32. The formulation of embodiment 29 or 30, wherein the *Listeria* strain is a from a freshly cultured *Listeria* stock.

[00479] 33. The formulation of any one of embodiments 29-32, wherein the formulation comprises 1% to 5% w/v sucrose.

[00480] 34. The formulation of embodiment 33, wherein the formulation comprises 2% to 3% w/v sucrose.

[00481] 35. The formulation of any one of embodiments 29-34, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00482] 36. The formulation of embodiment 35, wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00483] 37. The formulation of any one of embodiments 29-36, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00484] 38. The formulation of any one of embodiments 29-37, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the formulation comprises 2% to 3% w/v sucrose, and wherein the formulation does not comprise trehalose, MSG, or rHSA.

[00485] 39. The formulation of any one of embodiments 29-38, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame

encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00486] 40. The formulation of embodiment 39, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00487] 41. The formulation of embodiment 39, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

[00488] 42. A lyophilized composition produced by the method of any one of embodiments 1-28.

[00489] 43. A lyophilized composition comprising a *Listeria* strain, a phosphate buffer, and sucrose.

[00490] 44. The lyophilized composition of embodiment 43, wherein the lyophilized composition does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00491] 45. The lyophilized composition of embodiment 44, wherein the lyophilized composition does not comprise trehalose, MSG, or rHSA.

[00492] 46. The lyophilized composition of any one of embodiments 42-45, wherein the residual moisture in the lyophilized composition is between 1% and 5%.

[00493] 47. The lyophilized composition of embodiment 46, wherein the residual moisture in the lyophilized composition is between 2% and 4%.

[00494] 48. The lyophilized composition of embodiment 47, wherein the residual moisture in the lyophilized composition is between 3% and 4%.

[00495] 49. The lyophilized composition of any one of embodiments 42-48, wherein the lyophilized composition shows at least 60% viability after storage at –20°C or 4°C for 6 months.

[00496] 50. The lyophilized composition of embodiment 49, wherein the lyophilized composition shows at least 75% viability after storage at –20°C or 4°C for 6 months.

[00497] 51. The lyophilized composition of embodiment 50, wherein the lyophilized composition shows at least 80% viability after storage at –20°C or 4°C for 6 months.

[00498] 52. The lyophilized composition of any one of embodiments 42-51, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain.

[00499] 53. The lyophilized composition of any one of embodiments 42-52, wherein the *Listeria* strain is a recombinant *Listeria monocytogenes* strain, and wherein the lyophilized composition does not comprise trehalose, MSG, or rHSA, and wherein the residual moisture in the lyophilized composition is between 3% and 4%.

[00500] 54. The formulation of any one of embodiments 42-53, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

[00501] 55. The formulation of embodiment 54, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein.

[00502] 56. The formulation of embodiment 54, wherein the recombinant *Listeria* strain is an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.

BRIEF DESCRIPTION OF THE SEQUENCES

[00503] The nucleotide and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. The nucleotide sequences follow the standard convention of beginning at the 5′ end of the sequence and proceeding forward (i.e., from left to right in each line) to the 3′ end. Only one strand of each nucleotide sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. The amino acid sequences follow the standard convention of beginning at the amino terminus of the sequence and proceeding forward (i.e., from left to right in each line) to the carboxy terminus.

[00504] Table 3. Description of Sequences.

SEQ ID NO	Type	Description
1	DNA	SIINFEKL Tag v1
2	DNA	SIINFEKL Tag v2
3	DNA	SIINFEKL Tag v3
4	DNA	SIINFEKL Tag v4
5	DNA	SIINFEKL Tag v5
6	DNA	SIINFEKL Tag v6
7	DNA	SIINFEKL Tag v7
8	DNA	SIINFEKL Tag v8
9	DNA	SIINFEKL Tag v9
10	DNA	SIINFEKL Tag v10
11	DNA	SIINFEKL Tag v11
12	DNA	SIINFEKL Tag v12
13	DNA	SIINFEKL Tag v13
14	DNA	SIINFEKL Tag v14
15	DNA	SIINFEKL Tag v15
16	Protein	SIINFEKL Tag
17	DNA	3xFLAG Tag v1
18	DNA	3xFLAG Tag v2
19	DNA	3xFLAG Tag v3
20	DNA	3xFLAG Tag v4
21	DNA	3xFLAG Tag v5
22	DNA	3xFLAG Tag v6
23	DNA	3xFLAG Tag v7
24	DNA	3xFLAG Tag v8
25	DNA	3xFLAG Tag v9
26	DNA	3xFLAG Tag v10
27	DNA	3xFLAG Tag v11
28	DNA	3xFLAG Tag v12
29	DNA	3xFLAG Tag v13
30	DNA	3xFLAG Tag v14
31	DNA	3xFLAG Tag v15
32	Protein	3xFLAG Tag
33	Protein	Peptide Linker v1
34	Protein	Peptide Linker v2
35	Protein	Peptide Linker v3
36	Protein	Peptide Linker v4
37	Protein	Peptide Linker v5
38	Protein	Peptide Linker v6
39	Protein	Peptide Linker v7
40	Protein	Peptide Linker v8
41	Protein	Peptide Linker v9
42	Protein	Peptide Linker v10
43	Protein	PEST-Like Sequence v1
44	Protein	PEST-Like Sequence v2
45	Protein	PEST-Like Sequence v3
46	Protein	PEST-Like Sequence v4
47	Protein	PEST-Like Sequence v5
48	Protein	PEST-Like Sequence v6
49	Protein	PEST-Like Sequence v7
50	Protein	PEST-Like Sequence v8
51	Protein	PEST-Like Sequence v9

SEQ ID NO	Type	Description
52	Protein	PEST-Like Sequence v10
53	Protein	PEST-Like Sequence v11
54	Protein	PEST-Like Sequence v12
55	Protein	LLO Protein v1
56	Protein	LLO Protein v2
57	Protein	N-Terminal Truncated LLO v1
58	Protein	N-Terminal Truncated LLO v2
59	Protein	N-Terminal Truncated LLO v3
60	DNA	Nucleic Acid Encoding N-Terminal Truncated LLO v3
61	Protein	ActA Protein v1
62	Protein	ActA Protein v2
63	Protein	ActA Fragment v1
64	Protein	ActA Fragment v2
65	Protein	ActA Fragment v3
66	Protein	ActA Fragment v4
67	Protein	ActA Fragment v5
68	DNA	Nucleic Acid Encoding ActA Fragment v5
69	Protein	ActA Fragment v6
70	Protein	ActA Fragment v7
71	DNA	Nucleic Acid Encoding ActA Fragment v7
72	Protein	ActA Fragment Fused to Hly Signal Peptide
73	Protein	ActA Substitution
74	Protein	Cholesterol-Binding Domain of LLO
75	Protein	HLA-A2 restricted Epitope from NY-ESO-1
76	Protein	*Lm* Alanine Racemase
77	Protein	*Lm* D-Amino Acid Aminotransferase
78	DNA	Nucleic Acid Encoding *Lm* Alanine Racemase
79	DNA	Nucleic Acid Encoding *Lm* D-Amino Acid Aminotransferase
80	Protein	Wild Type PrfA
81	DNA	Nucleic Acid Encoding Wild Type PrfA
82	Protein	D133V PrfA
83	DNA	Nucleic Acid Encoding D133V PrfA
84	DNA	4X Glycine Linker G1
85	DNA	4X Glycine Linker G2
86	DNA	4X Glycine Linker G3
87	DNA	4X Glycine Linker G4
88	DNA	4X Glycine Linker G5
89	DNA	4X Glycine Linker G6
90	DNA	4X Glycine Linker G7
91	DNA	4X Glycine Linker G8
92	DNA	4X Glycine Linker G9
93	DNA	4X Glycine Linker G10
94	DNA	4X Glycine Linker G11
95	Protein	Detoxified Listeriolysin O (dtLLO)
96	Protein	Modified Cholesterol-Binding Domain of dtLLO
97	Protein	LLO Signal Sequence
98	Protein	ActA Signal Sequence
99	Protein	Variant FLAG Tag
100	Protein	10-Mer Peptide

EXAMPLES

Example 1. Representative Drug Substance Preparation and Lyophilization Cycle.

[00505] The typical current storage temperature for the ADXS-HER2 and ADXS-HPV (final liquid drug products) is −80°C, which interferes with the cold chain maintenance and poses supply chain challenges. Storage of *Listeria monocytogenes* final drug product (liquid) in a frozen state is inconvenient as the cold-chain must be strictly kept at all times to assure drug product efficacy and to avoid potential patient risks. ADXS-HER2, which is an attenuated, recombinant *Listeria monocytogenes* (*Lm*) transformed with a HER2/Neu fusion protein, is an *Lm* Technology™ immunotherapy product candidate being developed to target HER2 expressing cancers. Axalimogene filolisbac (ADXS-HPV) is an *Lm* Technology™ immunotherapy candidate developed for the treatment of HPV-associated cancers. It is an immunotherapy based on live attenuated *Listeria monocytogenes* that secretes fusion protein *Lm*-LLO-E7 targeting HPV-associated tumors. The storage of the final drug products in a frozen liquid state is inconvenient, as the cold chain must be kept at all times to assure drug efficacy and to avoid potential patient risks. Development of a lyophilization (lyo) cycle which favors the long term storage of the drug products at −20°C while maintaining the cold chain would be beneficial. Hence, a study was performed to develop a lyophilization cycle which favors the long term storage of the drug products.

Drug Substance Process Overview for ADXS-HPV Liquid Frozen Formulations

[00506] ADXS-HPV propagation was carried out entirely within a single use closed system provided by rocking wave motion bioreactor technology. The single-use closed system consists of a product 20 L culture bag for fermentation, a tangential flow filtration (TFF) for concentration and buffer exchange and a product manifold for DS container filling. Each of these components were sterilized by gamma irradiation. The drug substance manufacturing process flow diagram with in-process controls is shown in **FIG. 20.**

[00507] 1 M sodium hydroxide (NaOH) for pH control was prepared and sterile-filtered using two 0.2 µm filters in series into a 1 L pH control bag. The sterilizing filters were removed by cutting through a heat-sealed section of tubing. Fermentation media and pH control solution were prepared per **Table 4** and sterile-filtered through two 0.2 µm filters in series into a sterile 5

L media addition bag. The sterilizing filters were removed by cutting through a heat-sealed section of tubing.

[00508] Table 4. Fermentation Media Formulation.

Formulation	Components	Component Weights
Chloramphenicol Stock Solution	Chloramphenicol	0.68 g
	Ethanol, anhydrous	20 mL
Fermentation Media (5 L)	TSB	QS to 5 kg
	D (+) Glucose	32.54 g
	Chloramphenicol Stock Solution	5 mL
pH Control Solution	1M NaOH	1L
Overnight Culture	TSB	100 mL
	Chloramphenicol Stock	100 µL
Glucose Feed	D (+) Glucose	45.04 g
	WFI	250 mL

[00509] Diafiltration/wash buffer was prepared per **Table 5** and sterile-filtered through two 0.2 µm filters in series into a sterile 2×10 L bag. The sterilizing filters were removed by cutting through a heat-sealed section of tubing.

[00510] Table 5. Diafiltration Wash Buffer Formulation.

Formulation	Components	Component Weights
Formulation Buffer (20 L, 2×10 L)	Potassium dihydrogen phosphate (KH_2PO_4),	4.0 g
	Disodium hydrogen orthophosphate (Na_2HPO_4)	22.8 g
	Sodium chloride (NaCl)	160 g
	Potassium chloride (KCl)	4.0g
	Sucrose	400 g
	Water for Injection (WFI)	QS to 20.00 kg

[00511] A 20 L culture bag was pre-connected with probes for dissolved oxygen and pH monitoring. It was then aseptically filled with 5 L of growth medium. The media addition bag was then removed by cutting through a heat-sealed section of tubing.

[00512] The wave bag was inflated with sterile-filtered compressed O_2. Sterile-filtered compressed O_2 was continuously fed during propagation at a rate of 1 L/minute and removed through an outlet port. The rocking angle was set at 10° with a rocking rate of 18 per minute.

[00513] The pH control bag and the glucose feed bag were aseptically connected to the culture bag. During propagation, the process was automatically monitored and controlled for temperature, pH, and dissolved oxygen by an integrated controlling system.

[00514] An overnight culture was initiated from the WCB by pipetting 1mL of WCB into 100 mL of TSB and grown for approximately 12-16 hours until an OD600 of approximately 4. Then, 100 mL of the overnight culture was used to inoculate the production culture by aseptically transferring to the WAVE bag.

[00515] At four hours after inoculation, 200 mL of glucose was added to the culture. Growth proceeded to an OD_{600} between 7.5 and 8.5. This corresponds to approximately 1×10^{10} CFU/mL.

[00516] When the OD_{600} reached the target concentration, the culture bag was connected, using Readymates, to the sterile TFF manifold for concentration and diafiltration against the formulation buffer. The TFF module used a 0.2 μm pore size hollow fiber filter for low shear requirements of cell separation applications.

[00517] A peristaltic pump was used to feed the fermentation culture into the TFF system primed with formulation buffer. The bulk culture in the recirculation loop was set to a flow rate of 8 L/hr. The fermentation broth was concentrated 5-fold to a mass of approximately 1000 g.

[00518] The diafiltration/washing of the harvest concentrate was performed with ≥8 diavolumes (≥8 L). The harvest DS was sampled from the TFF assembly using a sampling manifold welded to the TFF. Each sample bag port was heat-sealed for removal.

[00519] The OD_{600} of the sample was measured and used to calculate the amount of dilution volume needed to reach an OD_{600} of 8.0 ± 0.5. The required amount of formulation buffer was pumped into the retentate bag to dilute the harvest to the required concentration. All volume transfers were controlled by weight change in the respective bags. The harvest was sampled and measured to confirm the required product concentration of 1×10^{10} CFU/mL was achieved. DS was sampled for QC analysis using the sampling manifold.

[00520] The DS was distributed into 1 L aliquots in four 1 L product bags with the fifth bag being filled with all the remaining DS. Each bag was heat-sealed for removal from the assembly. Each bag was individually labeled with the appropriate information and then stored at −80 ± 10°C.

[00521] Table 6. Drug Substance Processing Parameters.

Control Description	Operating Set-Point or Range
Fermentation Media pH	6.6–7.4
Rocking Rate	18 Rocks/minute
Rocking Angle	10°
Dissolved O_2 Set Point	35%

Drug Product Process Overview for ADXS-HPV Liquid Frozen Formulations

[00522] The manufacturing process of drug product (DP) involved dilution of the BDS to a final concentration of 1×10^9 CFU/mL and aseptic filling of the formulated axalimogene filolisbac into sterile 4 mL glass vials, stoppering with 13 mm chlorobutyl stoppers and over-sealing with aluminum flip-off seals with polypropylene discs. The process flow diagram is shown in **FIG. 21.**

[00523] Frozen drug substance (DS), in 1 L bags, containing up to 5 L in 1 L aliquots, was stored at −80 ± 10°C until manufacture of DP. DS was thawed at ambient temperature with a target of 3 hours to initiate manufacture of DP.

[00524] Under Grade A conditions, up to 5 L was aseptically transferred via a pump into a dedicated sterile glass carboy assembly. The pooled bulk material in the carboy was stirred from 80-300 rpm during the material transfer and was then connected to the sterile disposable filling module using a sterile tube welder. For the proposed commercial process a 1:10 dilution step was performed with final formulation buffer to a targeted CFU of 1×10^9 CFU/mL.

[00525] Depyrogenated 4 mL (DIN 2R Type I borosilicate) glass vials were semi-automatically filled by a peristaltic pump with 1.2 mL of DS using a sterile, single use filling line and filling needle. Filled vials were immediately stoppered with sterilized chlorobutyl stoppers. During filling, the fill volume was controlled by weight checks on 1 vial in every 300 ± 50 filled vials.

[00526] The finished vials were over sealed with aluminum crimp flip-off seals with polypropylene discs. The vials were externally wiped with a 0.35% acetic acid solution and were transferred to a Grade D room for 100% visual inspection.

[00527] Vials were visually inspected for container-closure defects or atypical appearance of product. Bulk packaged vials were stored at −80 ± 10°C until shipping to the labeling and packaging site.

[00528] Table 7. Drug Product Process Parameters

Process Stage	In-Process Control Description	Set Point or Operating Range
Bulk mixing	Stirring speed sample bottle, > 3 L volume	190–300 rpm
Bulk mixing	Stirring speed sample bottle, 2-3 L volume	140–240 rpm
Bulk mixing	Stirring speed sample bottle, 1-2 L volume	100–160 rpm
Bulk mixing	Stirring speed sample bottle, 0-1 L volume	80–120 rpm
Aseptic filling	Average line speed	1300 ± 200 vials/hr
Aseptic filling	Adjustment filling volume	1.2 mL
Aseptic filling	Filling weight max.	1.279 g
Aseptic filling	Filling weight min.	1.181 g
Aseptic filling	Reverse impulse setting (peristaltic dosing system)	2–3
Aseptic filling	Acceleration setting (peristaltic dosing system)	200
Aseptic filling	Timer delay setting (peristaltic dosing system)	0.3 s
Over sealing	Average line speed	300 ± 50 vials/hr
Over sealing	Stopper safety setting	12.2 cm
Over sealing	Capping station vertical height	9.6 cm
Over sealing	Capping station horizontal height	3.2 cm
Over sealing	Closure station top dead center	7.2 cm
Over sealing	Closure station bottom dead center	4.2 cm

[00529] Table 8A. Specifications for *Axalimogene Filolisbac* Drug Product.

Test	Test Type	Acceptance Criteria
Identification		
Western Blot for HPV16-E7	In-house method	Principal bands conform to reference standard
Western Blot for tLLO	In-house method	Principal bands conform to reference standard
Purity		
Microbiological Examination	In-house method based on Ph. Eur. 2.6.12, 2.6.13	Single species
Potency		
J774 Infectivity	In-house method (Cell based plate assay)	$\geq 1\times10^{3}$ CFU/mL
Content		
Viable Cell Count	In-house method	3×10^{8} CFU/mL - 5×10^{10} CFU/mL
General		
Appearance	Based on USP <1>	Free-flowing cream-colored suspension
Osmolality	Ph. Eur. 2.2.35	280 to 420 mOsm/kg
pH	Ph. Eur. 2.2.3	6.0 to 7.9
Safety		
Endotoxin	Ph. Eur. 2.6.14	≤ 35 EU/mL

[00530] Table 8B. Specifications for *Axalimogene Filolisbac* Drug Substance.

Test	Test Type	Acceptance Criteria
Identity		
Western blot for HPV16-E7[1]	In-house method	Principal bands conform to reference standard
Western blot for tLLO[1]	In-house method	Principal bands conform to reference standard
prfA Deletion[1]	In-house method (PCR)	*prfA* gene is not present on the chromosome
prfA Mutation[1]	In-house method (PCR)	Point mutation in *prfA* gene is present on the plasmid
Purity		
Microbiological Examination[2]	In-house method based on Ph.Eur. 2.6.12 and Ph.Eur. 2.6.13	Single species (Negative for the presence of Bacterial and Fungal contamination)
Percentage of Viable cells	In-house method (Fluorometric plate assay)	≥60% Viable cells
Potency		
J774 Infectivity	In-house method (cell-based plate assay)	≥ 1×10^3 CFU/mL
Content		
Extractable Volume[1]	Based on USP <1>	≥1 mL
Viable Cell Count	In-house method	3×10^8 CFU/mL - 5×10^{10} CFU/mL
Plasmid Copy Number[1]	In-house method	5 to 100 copies
General		
Appearance	Based on USP <1>	Free-flowing cream-colored suspension
Osmolality[1]	Ph. Eur. 2.2.35	280 to 420 mOsm/kg
pH	Ph. Eur. 2.2.3	6.0 to 7.9
Safety		
Antibiotic Sensitivity[1]	In-House method	Resistant to 50 µg/mL streptomycin Resistant to 34 µg/mL chloramphenicol Sensitive to 0.25 µg/mL ampicillin Sensitive to 1 µg/mL tetracycline Sensitive to 1.5 µg/mL ciprofloxacin
Reversion of *prfA* Mutation[1]	In-house method (PCR)	No detectable reversion of point mutation in *prfA* gene on the plasmid
Endotoxin[2]	Ph. Eur. 2.6.14	≤ 35 EU/mL
Container Closure Integrity[3]	USP<1207>	No ingress of dye

Notes
[1] Tested only at release
[2] Tested at release and at the end of shelf life
[3] Tested for stability only
PCR = polymerase chain reaction, Ph. Eur. = European Pharmacopoeia, USP = United States Pharmacopeia.

Lyophilization as an Alternative

[00531] Drug product can also be lyophilized for long-term storage. Vials with drug product were loaded onto lyophilizer shelves that have been chilled to a refrigerated temperature, in some embodiments, about 4°C. The chamber door was closed, and the vials were cooled to a

temperature just above the freezing point of the formulation by reducing the shelf temperature to around –4°C and holding it there for about 30 minutes. The formulation was then frozen by ramping the shelf temperature at a rate of approximately 0.5°C/min to a temperature between –40°C and –50°C, or about –45°C, and maintaining that temperature for several hours until all vials were frozen and the product temperatures were close to the shelf temperature. To conduct primary drying, the chamber was evacuated, and the pressure was maintained at about 0.09 mbar with sterile nitrogen. The shelf temperature was raised at about 1°C/min to a temperature between –18°C and –22°C, or about –18°C, and maintained at that value until all product temperatures exceeded the shelf temperature for a minimum of about 10 hours. To conduct secondary drying, the shelf temperature was raised at about 0.2°C/min to a final value of 20°C and kept there for at least 2 hours, while the pressure was maintained at about 0.09 mbar. At the end of this secondary drying time, the shelf temperature was reduced to about 10°C, then the pressure was increased to about 500 mbar with nitrogen, and the vials were stoppered within the lyophilizer. A representative lyophilization cycle is shown in **Table 9**.

[00532] Table 9. Representative Lyophilization Cycle.

Step	Duration [hh:mm]	Shelf Temp. [°C]	Vacuum [mbar]
Loading	N/A	4	Off
Freezing	00:20	–4	Off
	00:30	–4	Off
	01:30	–45	Off
	02:00	–45	Off
Primary drying	N/A	–45	0.090
	00:30	–45	0.090
	00:18	–18	0.090
	26:00*	–18	0.090
Secondary drying	03:10	20	0.090
	02:00	20	0.090
End of cycle	00:10	10	0.090
	N/A	10	0.090
Stoppering	N/A	10	500
* Until all product temperature probes have been above shelf temperature for at least 10h.			

Example 2. Optimization of Lyophilization Parameters for *Listeria monocytogenes*.

[00533] The ADXS *Listeria monocytogenes* (*Lm*) drug products are currently formulated in a phosphate buffered saline (KH_2PO4, Na_2HPO_4, KCl, NaCl) containing 2.0% sucrose with recommended storage conditions of -80°C. The ultra-low storage temperature poses challenges for cold chain maintenance and supply chain. Hence, a development program was initiated for

the development of a stable lyophilized drug product (DP). The goal of the program was to develop a lyophilization (Lyo) process which favored the long-term storage of the drug products at –20°C or 2-8°C. A series of experiments were performed with different parameters to develop and optimize the formulation, the pre-conditioning of cells, storage/handling of the drug substance and the lyophilization cycle. Through optimization of various parameters, a stable lyophilized formulation was developed which has demonstrated long-term (18 months) stability at both 2-8°C and –20°C.

[00534] Parameters tested in the experiments below include formulation parameters (buffer composition (the solution in which the cells are lyophilized in), excipient composition (the inactive substance used to aid in stability), and OD_{600} at the time of lyophilization),

[00535] A series of experiments were performed with different test parameters to develop and optimize the formulation, the pre-conditioning of cells, and the lyophilization cycle. Formulation parameters tested included buffer composition (the solution in which the cells are lyophilized in), excipient composition (the inactive substance used to aid in stability), and OD_{600} at the time of lyophilization. Preconditioning of cell parameters tested included fresh/frozen (the storage condition of the drug substance before lyophilization), induction of stress response prior to lyophilization (shift in pH and/or temperature), and drug substance hold time/temperature (conditions at which drug substance is thawed and held prior to lyophilization). Lyophilization cycle parameters tested included primary drying shelf temperature (heat input for sublimation of the frozen water), secondary drying shelf temperature (heat input for desorption of moisture remaining after primary drying), and addition of an annealing step (heating the frozen formulation to a temperature below 0°C to allow rearrangement of the ice pore structure and possibly improve primary drying). Outcomes measured to assess the success of a lyophilization run included viable cell count (VCC) over time and under different conditions (stability at -80°C, stability at 2-8°C, stability at –20°C, and accelerated stability at 30°C), residual moisture, and reconstitution time.

[00536] The experiments described below identified several findings that appeared to enhance the stability of the lyophilized product: (1) higher residual moisture improved the stability of the lyophilized product (WP7-Lyo4); (2) a higher shelf temperature during primary drying improved the stability of the lyophilized product (WP7-Lyo9); (3) preconditioning of the cells prior to lyophilization through heat shock improved the stability of the lyophilized product (WP7-Lyo5);

(4) higher VCC demonstrated slight improvement in stability of the lyophilized drug product relative to lower VCC (WP7, Cycle 3); and (5) the data demonstrate that the storage of the drug substance in a 1L LDPE bag and thawing at 37°C prior to lyophilization improved stability of the lyophilized drug product (WP7, Cycle 3). The data show that a lyophilized drug product that is stable at both –20°C and 2-8°C long term has been successfully developed. The resulting drug product demonstrated good stability at both accelerated and intended storage conditions and low loss in potency due to lyophilization.

[00537] To identify and characterize lead formulations, Lyo1 and Lyo2 experiments were performed, which led to the identification of two phosphate-based formulations with 5% sucrose and with 5% sucrose plus amino acid (AA) mix (final concentrations: 36 mM arginine, 57 mM glutamic acid, and 7 mM isoleucine). The characterization of these lead formulations showed their critical temperatures to be close together, allowing for the development of one cycle for both formulations. The residual moisture targeting and evaluation experiments, Lyo3 and Lyo4, showed the best results for higher moisture levels at a sucrose level of 2.5% and allowed for the optimization of the sucrose level at 2.5% for further cycle development.

[00538] The three main areas of evaluation for the lyophilization cycle development study included: (1) formulation development for the screening of buffer and excipient; (2) cell culture development for the pre-conditioning of the cells prior to lyophilization; and (3) optimization of the lyophilization cycle for targeted residual moisture (RM). The series of experiments performed in the lyophilization (lyo) cycle development with the different parameters tested are summarized in **Table 10**.

[00539] Table 10. Summary of Experiments Performed in Lyophilization (Lyo) Cycle Development

Process Step	Expt. No.	Expt. Description	Construct	Buffer	Excipients	OD	Primary Drying Shelf Temp. (°C)	Secondary Drying Shelf Temp. (°C)	Temp/ pH Shift	DS Hold	% RM	Conditions Evaluated	Results and Conclusions
Formulation	Lyo1	Buffer Screen	HER2	Citrate Phosphate MOPS	Sucrose, Trehalose, MSG, rHSA	10	−12	20				2-8°C, accelerated stability conditions 25°C for 3 days.	Two phosphate-based formulations with 5% sucrose and 5% sucrose+AA mix performed well. These 2 formulations were used for further cycle development.
	Lyo2			Phosphate	Sucrose, rHSA, AA mix (amino acid mix)	10, 2	−12	20					
		Characterization of Tc, Tg, and Tg′	HER2	Phosphate	5% sucrose 5% sucrose + AA mix	10	−22	20					The critical temperatures were close together. Development of one cycle for both formulations.
Lyo cycle	Lyo3	Residual moisture targeting	HER2	Phosphate	5% sucrose 2.5% sucrose	10	−22	20					
	Lyo4	Evaluation of residual moisture on stability	HER2	Phosphate	5% sucrose 2.5% sucrose	10	−22	20			~5%, ~3%, and 1%	2-8°C, accelerated stability conditions for 1, 2, and 3 days at 30°C.	Best results were at higher moisture levels for the sucrose level of 2.5%. The sucrose level was fixed at 2.5% and the moisture level was fixed at 3.5%.
Cell culture/ pre-conditioning of cells	Lyo5	Evaluation of stress treatments pre-lyophilization on stability	HER2	Phosphate	2.5% sucrose	10	−22	20	*Group 1*: control; *Group 2*: temp-shift; *Group 3*: pH-shift; *Group 4*: pH- and temp-shift		3.5%	2-8°C, accelerated stability conditions for 1, 2, and 3 days at 30°C.	
	Lyo6	Evaluation of temp-shift pre-lyophilization on stability	HPV	Phosphate	2.5% sucrose	10	−22	20	Only temp-shift, no control		3.5%		

Process Step	Expt. No.	Expt. Description	Construct	Buffer	Excipients	OD	Primary Drying Shelf Temp. (°C)	Secondary Drying Shelf Temp. (°C)	Temp/ pH Shift	DS Hold	% RM	Conditions Evaluated	Results and Conclusions
Lyo cycle	Lyo7	Evaluation of temp-shift pre-lyophilization on stability	HPV	Phosphate	2.5% sucrose	10	−30	20	Only temp-shift, no control		3.5%	2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	Significant losses on accelerated stability with the decreased shelf temperature.
Cell culture/ pre-conditioni	Lyo8	Hold time study	HPV	Phosphate	2.5% sucrose	10	−22			*Part A*: fresh *Part B*: frozen hold		2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	Part A showed better stability profile under accelerated conditions than Part B.
Lyo cycle	Lyo9	Evaluation of increased primary drying shelf temperature	HPV	Phosphate	2.5% sucrose	10	−18	20	Only temp-shift, no control		3.5%	2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	Improvement in accelerated stability was observed with increased shelf temperature.
	Lyo10	Comparison of plus/minus temperature shift	HPV	Phosphate	2.5% sucrose	10	−18	20	*Part A*: without temp-shift *Part B*: with temp-shift		3.0%	2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	The results were comparable for with and without the temperature shift.
	Lyo11	Stability study without temperature shift	HPV	Phosphate	2.5% sucrose	10	−18	20	Without temp-shift		2.5%-3.0%	2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	
Cell-culture/ pre-conditioning of cells	Lyo12	Stability study of fresh vs. frozen material using different thawing	HPV	Phosphate	2.5% sucrose	10	−18	20	Without temp-shift	*Part A*: fresh *Part B*: frozen, thawed at 2-8°C *Part C*: frozen, thawed at 37°C and incubated 4 h	2.5%-3.0%	2-8°C, −20°C, and accelerated stability conditions for 1, 2, and 3 days at 30°C.	
Cell-culture/ pre-conditioning of cells	Lyo13	Stability study of Fresh vs Fresh/formulated material stored 3 days at 2-8°C	HPV	Phosphate	2.5% sucrose	10	−18	20	Without temp-shift	*Part A*: Fresh; *Part B*: Stored 3 days at 2-8°C	2.5-3.0%	2-8°C, −20°C and Accelerated conditions for 1, 2, and 3 days and 30°C	

Process Step	Expt. No.	Expt. Description	Construct	Buffer	Excipients	OD	Primary Drying Shelf Temp. (°C)	Secondary Drying Shelf Temp. (°C)	Temp/ pH Shift	DS Hold	% RM	Conditions Evaluated	Results and Conclusions
Lyo Cycle	Lyo14	Evaluation of commercial presentation in 2R vials	HPV	Phosphate	2.5% sucrose	1.5	−18	20	Without temp-shift		2.5-3.0%	2-8°C, −20°C and Accelerated conditions for 1, 2, and 3 days and 30°C	
Cell-culture/Pre-conditioning of cells	Lyo15	Stability study of Fresh vs. Frozen pellet/reconstituted material in 2R vials	HPV	Phosphate	2.5% sucrose	1.8	−18	20		Suspension A: Fresh; Suspension B: Frozen, thawed at 37°C and resuspended	2.5-3.0%	2-8°C, −20°C and Accelerated conditions for 1, 2, and 3 days and 30°C	

Description of Experiments

2.1. Screening and Characterization of a Lyophilization Formulation.

[00540] For the identification, optimization and characterization of 2-3 lead formulations with good stability that could be continued with during further cycle development, two lyophilization experiments (Lyo1, Lyo2) were executed and 6-month stability data was generated. The lead formulations from this study were then characterized for their critical temperatures Tc (collapse temperature), Tg′ (glass transition temperature of the frozen formulation), and Tg (glass transition temperature of the lyophilized product).

[00541] The formulations used in Lyo1 were citrate-, phosphate- and MOPS-based formulations. The formulations used in Lyo2 were only phosphate-based formulations because phosphate-based formulation had better performance compared to citrate- and MOPS-based buffers and required the smallest process change as they were closest to the current drug substance formulation.

2.1.1. WP5-Lyo1.

[00542] **Materials and Methods.** The ADXS-HER2 drug product was used for this study. OD_{600} = 10 was evaluated. The buffers and excipients used in the formulations were as follows: Three different buffers were used in the formulations: citrate; phosphate; and MOPS (3-(*N*-morpholino)propanesulfonic acid). The stabilizer mix components (excipients) used in the formulations included various combinations of sucrose, trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

[00543] **Study Design.** Three different pH buffers combined with 6 different excipient combinations resulted in 18 different formulations. For each formulation, 20× 6R vials were filled with 2 mL, resulting in a cake height of ~6.54 mm. The 360 vials were randomly distributed on the 3 shelves of the lyophilization machine to average out edge effects. The lyophilization run was completed after ~44h and 30 min. Vials were closed with 0.2 µm filtered air at 600m bar. The vials were transferred to 2°C-8°C storage and crimped. Residual moisture was measured directly after lyophilization and after 6 months storage. Viable cell count (VCC) before and after lyophilization and the corresponding % survival data was analyzed. The spread plate method was used for determining the total viable cell count of microorganisms present per mL of cell culture. The medium used to perform viable cell count may vary and is determined

by the growth requirements of the organism. *Listeria monocytogenes* samples were cultured in Trypticase Soy Agar (TSA).

[00544] **Results.** Phosphate- and citrate-based buffers yielded comparable recovery results. *See* **FIG. 1**. Residual moisture (RM) analysis revealed that the addition of MSG increased the RM in all buffer systems by ~1.0%-1.5%. Without MSG, the RM values ranged from ~1.8% to ~3.0%. There was no clear tendency for increase or decrease of % RM, indicating inter-assay variability

[00545] Multivariate data analysis (MVDA) confirmed the decision to continue formulation development based on phosphate as there was no clear superiority of one buffer system, and phosphate requires only modest process change. *See* **FIG. 1**. The regression lines using stability at 4°C showed the most consistency under the 5:0:0:0 stabilizer mix combination (sucrose:trehalose:MSG:rHSA). *See* **FIG. 1**.

[00546] MVDA analysis also showed that in the accelerated stability study, the samples stored at 30°C for 3 days showed a comparable recovery as the samples stored at 4°C for 6 months. *See* **FIG. 2A** and **2B**. This indicated that higher storage temperature resulted in faster loss of viability and that accelerated conditions might be predictable data for long term storage at 2°C-8°C. The lyo-cake appearance was good overall with no major defects or melt-backs. The sucrose based formulations showed slight cake shrinkages at the edges (top-crown and bottom of the vials). The change in VCC at 3 days at 30°C was similar to that seen at 6 months at 4°C. **FIG. 22A** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization and post-lyophilization after storage at different temperatures for different amounts of time in the Lyo1 experiment. Accelerated stability for 3 days at 30°C was similar to 6 months stability at 4°C. **FIG. 22B** shows residual moisture immediately after lyophilization and after 6 months at 2-8°C in the Lyo1 experiment.

2.1.2. WP7-Lyo2.

[00547] **Materials and Methods.** The ADXS-HER2 drug product was used for this study. OD_{600} values (OD in vial is representative of cell concentration) ranging from 2 to 20 were evaluated, and two different final OD_{600} values were evaluated: OD_{600} = 10 (same as Lyo1); and OD_{600} = 2.0. The buffer used was phosphate-based buffer, and the stabilizer mix components (excipients) used included sucrose, amino acid (AA) mix, and rHSA.

[00548] **Study Design.** One pH buffer, 9 different excipient combinations, and 2 bacterial concentrations resulted in 18 different formulations. The lyophilization run was performed similar to Lyo1. VCC was assessed on samples before lyophilization, on frozen samples (−80°C) before lyophilization, on samples after lyophilization, and on samples after lyophilization and after storage for 1, 2, 3, 6, 9, 12, or 18 months at 2-8°C, −20°C, and −80°C.

[00549] **Results.** In both the OD_{600}=2 and OD_{600}=10 groups, the highest recoveries were with sucrose-only formulations: 2.5% in OD_{600}=10; and 5% sucrose in OD_{600}= 2.0. Parallel to Lyo1, there was a correlation between formulation and residual moisture. Formulations containing sucrose-only or a mixture of sucrose + AA had better residual moisture compared to rHSA formulations, which were drier. Percent RM at 6 months compared to after lyophilization was increased for all samples. MVDA analysis showed that rHSA had detrimental effects on stability. Highest recoveries (and lowest variability) were observed at lowest sucrose concentration (2.5%) and with higher OD_{600} values (10 versus 2). The lyo-cake appearance was similar to Lyo1, and there was no significant difference between the two OD_{600} vials. **FIG. 3** shows VCC data for different OD levels and stabilizer combinations (OD, stabilizer). OD levels of 2.0, 3.0, 10.0, 12.5, 15.0, 17.5, and 20.0 were tested. Stabilizers including 2% and 5% sucrose were tested, optionally in combination with AA mix. VCC as a percentage of the count before lyophilization showed similar slopes at all OD levels and stabilizer combinations. Lyophilization samples stored at −20°C for 1 month showed VCC results in between. **FIG. 23A** shows VCC data (percent of average pre-lyophilization VCC) before lyophilization and post-lyophilization after storage at different temperatures for different amounts of time (months) in the Lyo2 experiment. **FIG. 23B** shows residual moisture immediately after lyophilization and after 6 months at 2-8°C in the Lyo2 experiment.

[00550] Table 11. Summary of Conditions in Figure 23B.

Target	Buffer #	OD_{600} in Vial	OD_{600} of 2-fold Stock	#6R Vials	Fill Volume (mL)	Formulations	X-fold increase in CFU/vial compared to WP5
High OD_{600} Formulation	1	12.5	25	40	3	Phosphate + 5% Sucrose	1.88 (compared to OD_{600}=10)
	2	12.5	25	40	3	Phosphate + 5% Sucrose + Amino Acid	
	3	10	20	40	3	Phosphate + 5% Sucrose	1.50 (compared to OD_{600}=10)
	4	10	20	40	3	Phosphate + 5% Sucrose + Amino Acid	
Low OD_{600} Formulation	5	3	6	40	3	Phosphate + 5% Sucrose	2.25 (compared to OD_{600}=2)
	6	3	6	40	3	Phosphate + 5% Sucrose + Amino Acid	
	7	2	4	40	3	Phosphate + 5% Sucrose	2.25 (compared to OD_{600}=2)
	8	3	4	40	3	Phosphate + 5% Sucrose + Amino Acid	
OD_{600} Maximum Evaluation	9	20	40	12	3	Phosphate + 5% Sucrose	3.00 (compared to OD_{600}=10)
	10	17.5	35	12	3	Phosphate + 5% Sucrose	3.63 (compared to OD_{600}=10)
	11	15	30	12	3	Phosphate + 5% Sucrose	2.25 (compared to OD_{600}=10)

[00551] Conclusions. During this study, 18 different formulations in Lyo1 and 9 different formulations in Lyo2 were analyzed. Two formulations from Lyo1 and Lyo2 were identical, hence a total of 18+7=25 different formulations were analyzed (formulation-1 of Lyo1 is formulation-2 of Lyo2 and formulation-5 of Lyo1 is formulation-6 of Lyo2). Based on VCC results from Lyo1, optimization in Lyo2 was performed with phosphate-based formulations based on superior performance of phosphate and only modest change to current process. The lead formulation of Lyo1 was 5% sucrose, which performed better or equally well compared to the other buffers in Lyo2. In Lyo2, an amino acid mixture was expected to exhibit additional protective effects during long-term storage. Both the lead formulation of Lyo1 (5% sucrose) and the formulation with amino acids were then characterized for their critical temperatures (Tc, Tg, and Tg′), which were found to be close together, allowing for the development of one single cycle for both formulations.

2.2. Residual Moisture Targeting.

[00552] To obtain information about the process of drying samples during lyophilization cycle and establish correlation between secondary drying and residual moisture content, the Lyo3 experiment was performed. This enabled targeting for specific residual moisture contents in future lyophilization experiments.

2.2.1. WP5-Lyo3.

[00553] **Materials and Methods.** The ADXS-HER2 drug product was used for this study. The formulations used were phosphate-based with 2.5% sucrose, 5% sucrose, and 10% sucrose. The stabilizer mix included different combinations of sucrose, AA mix, and rHSA. Two different OD_{600} values were evaluated: OD_{600} = 10 and OD_{600} = 2.0.

[00554] **Study Design.** Stability was tested at 4°C for 13 days, 1 month, 3 months, and 6 months. Accelerated stability was tested using samples stored at 13 days at 4°C before accelerating at 30°C for 1 day, 2 days, or 3 days. VCC was measured before lyophilization, VCC and RM were measured after lyophilization and on stability, and RM was measured after lyophilization.

[00555] To obtain information about the process of drying, the cycle was aborted at different time points to take samples to analyze residual moisture (RM). The first samples were taken directly at the end of primary drying. A heating rate of 0.2°C/min was used in the ramp to secondary drying, and further samples were taken directly after the ramp to secondary drying. Secondary drying was performed at +20°C for 8h (3h longer than in Lyo2). Samples were taken every 2h and analyzed immediately. Based on the live RM data, secondary drying might be extended if the target of <1% is not reached.

[00556] **Results/Conclusions.** As shown in **FIG. 4**, it is clear from the raw VCC levels that an increase in rHSA is associated with instability, counts are higher at OD_{600} = 10, and the lowest variability of results is seen at the low level of sucrose (2.5%). This is reinforced by 6-month data.

[00557] As shown in **FIG. 5**, the moisture results show little distinction based on OD, lower RM with increased rHSA at sucrose levels of 10% and 5%, and lowest variability of results at the lowest level of sucrose (2.5%) and without rHSA.

[00558] These experiments also showed that material with RM within a desired range of ~5% to <1% can be generated by taking the product out of the freeze-dryer at distinct process steps, including the end of primary drying, the end of the ramp to secondary drying, and different time points during secondary drying.

2.3 Evaluation of Optimal Residual Moisture on Stability.

[00559] To optimize the target residual moisture and recovery after lyophilization, the Lyo4 stability study was performed.

2.3.1 WP7-Lyo4.

[00560] Data generated under WP7-Lyo3 demonstrated proof of principle that material with Residual Moisture within the targeted range of ~5% to <1% can be generated by removing samples from the freeze-dryer at distinct process steps: (1) end of primary drying; (2) end of ramp to secondary drying; and (3) at different time points during secondary drying. Based on the initial data material for long-term stability studies at 2-8°C and at 30°C, 65%RH for short-term accelerated stress stability, was generated. The % residual moisture (RM) targets were ~5%, ~3% and about 1%. Based on all data available that was available at the time material was generated for two lead formulations: (1) phosphate buffer, pH 7.2, 5% sucrose; and (2) phosphate buffer, pH 7.2, 2.5% sucrose.

[00561] **Materials and Methods.** The ADXS-HER2 drug product was used for this study. The formulations used were phosphate-based with 2.5% sucrose and 5% sucrose. OD_{600} values were OD_{600} = 20 (~$2x10^{10}$ CFU/mL). Moisture levels were controlled by removing the samples out of the freeze-dryer at distinct process steps: (1) end of primary drying (residual moisture target ~5%); (2) end of ramp to secondary drying (residual moisture target ~3%); and (3) end of secondary drying (residual moisture target ~1%).

[00562] **Study Design.** The lyophilization run was performed similar to Lyo3 except initial OD value was 20. Bacteria were cultivated and concentrated by centrifugation as described in Lyo2 to an OD_{600}~20 (~$2x10^{10}$CFU/mL). Moisture levels were controlled by where the samples were taken in the lyophilization cycle: (1) high moisture; after primary drying; moistures ~5.4-5.8%; (2) mid moisture; after ramp (immediately after the ramp of the shelf temperature from the primary drying temperature to the secondary drying temperature); moistures ~3.7-4.5%; and (3)

low moisture; after secondary drying; moistures ~1.1-1.3%. The first samples were taken directly at the end of primary drying (RM target is ~5%), and the second lot of samples were taken directly after the ramp to secondary drying (RM target is ~3%). Secondary drying was performed for 12h, after which the third lot of samples were removed (RM target is ~1%). After removal, all vials were crimped and stored at 2-8°C (including vials for later stress stability study). RM and VCC before lyophilization, after lyophilization, and at accelerated conditions for 1, 2 and 3 days (30°C) were analyzed. VCC titer was measured, as both count and percent of count before lyophilization.

[00563] **Results.** Moistures achieved in this study bracket moistures seen before. Combining Lyo4 and Lyo2 studies, focusing only on sucrose 2.5% and 5.0%, and removing all reference to studies with rHSA, comparability at accelerated conditions was seen. Best results were at higher moisture levels for the sucrose level of 2.5%. As shown in **FIG. 6**, RM achieved in this study "bracket" moistures previously seen. RM results are shown as both the individual values and the average. The dotted lines outline the range (high and low) of results that had been seen in the long-term study on Lyo2. The comparability at accelerated conditions is shown in **FIGS. 7A**, **7B**, **24A**, and **24B**. As shown in **FIGS. 7A** and **7B**, the best results are at higher moisture levels for sucrose levels of 2.5%. **FIG. 24A** shows residual moisture (RM) using 2.5% sucrose after primary drying, after ramp, and after secondary drying after storage at different temperatures for different amounts of time in the Lyo4 experiment. **FIG. 24B** shows residual moisture (RM) using 5.0% sucrose after primary drying, after ramp, and after secondary drying after storage at different temperatures for different amounts of time in the Lyo4 experiment.

[00564] **Conclusions.** This study confirmed that higher residual moisture (RM) resulted in a better VCC profile under accelerated conditions at $OD_{600} = 20$. The best VCC profiles obtained on accelerated stability were at higher moisture levels for the sucrose level of 2.5%. Hence, the sucrose level was fixed at 2.5% and the target residual moisture level for future development experiments was targeted at 2.5-3.5%.

2.4. Evaluation of Stress Treatments Pre-Lyophilization.

[00565] To evaluate stress treatments pre-lyophilization and its effects on stability of the lyophilized material, Lyo5 was performed. This study simulated stress conditions potentially experienced during the fermentation process.

[00566] Cold shock, heat shock, and osmotic shock to cells, may induce expression of genes involved in the general stress response. The genetic response to these shock conditions are necessary for defending the cell against stress damage and death. Thus, greater cell survival during lyophilization may be achieved by activating the stress response. To evaluate the induction of a stress response in the *Lm* prior to lyophilization and its effects on stability of the lyophilized material, WP7-Lyo5 was performed. This study induced a stress response either by a pH shift or cold shock prior to formulation and lyophilization.

2.4.1. WP7-Lyo5.

[00567] The study goal for WP7-Lyo5 was to evaluate if induction of a stress response (cold shock and pH shift) in the *Lm* could improve viability in the reconstituted drug product.

[00568] **Materials and Methods.** The ADXS-HER2 drug product was used for this study. The experiment comprised of the following four arms: (1) Group-1: control culture; (2) Group-2: temperature-shift culture; (3) Group-3: pH-shift culture; and (4) Group-4: pH- and temperature-shift culture (pH-shift first followed by temperature-shift). To achieve the temperature-shift and pH-shift, immediately after harvesting the cells from the bioreactor, the cells were either placed in an ice bath or the pH was dropped by the addition of acid. This induces a stress response in the cells that activates a set of genes that seem to better prepare the cells for lyophilization. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.5% and the OD_{600} = 10 (~$1x10^{10}$CFU/mL). 6R vials were filled with approximately 2mL of Drug Product. Accelerated stability was evaluated at 30°C for 1, 2, and 3 days.

[00569] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, materials for group-1 (control) and group-2 (temperature-shift) were removed. Material for group-1 was processed further until formulated bulk (bulk drug substance) was obtained, which was then stored at 2-8°C until vial filling. For group-2, the temperature-shift was performed in an ice/salt/water bath, after which the material was stored at 2-8°C for 30 min. Then the material was processed further until formulated bulk was obtained, which was stored at 2-8°C until vial filling. In the meantime, the pH-shift was performed in the bioreactor using 2M HCl to pH=5.25. Then the material for group-3 (pH-shift) and group-4 (pH-/temp-shift) was removed. Material from group-3 was processed further until formulated bulk was obtained and was stored at 2-8°C until vial filling. For group-4, the temperature-shift was performed as described above

and the material was stored at 2-8°C for 30 min. Then the material was processed further until formulated bulk was obtained. Once formulated bulks for all groups were obtained, VCC was analyzed and lyophilization was initiated.

[00570] The lyophilization run was performed similar to Lyo4. For a target residual moisture of 3.5%, 2h secondary drying time was used. VCC was analyzed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C). VCC titer was measured, expressed as both count and percent of count before lyophilization.

[00571] **Results.** The data at 3 and 6 months (and 9, 12, and 18 months) at both –20°C and 2-8°C demonstrate good comparability across all four groups. *See* **FIG. 8** (VCC) and **FIG. 25** (RM). **FIG. 8** shows the percentage of VCC after lyophilization for each experimental condition (pH shift, temperature shift, pH/temperature shift and control) evaluated on stability at –20°C, 2-8°C, and accelerated stability. **FIG. 25** shows residual moisture levels at T=0 for each experimental condition (pH shift, temperature shift, pH/temperature shift, and control) evaluated on stability at 2-8°C. The temperature shift condition showed a more stable profile on long term stability compared to control or pH shift.

[00572] The data demonstrated a good stability profile both at –20°C and 2-8°C for the temperature shift sample relative to the other arms of the study. There does not appear to be a benefit to the pH shift or the pH + temperature shift pre-treatment. The data indicate that preconditioning the cells prior to lyophilization may increase the long-term stability of the product. The data do not show any clear trends in Residual Moisture across treatment arms or upon long-term storage.

[00573] **Conclusion**. This study showed that a more stable profile on long term stability and accelerated stability conditions was obtained with the temperature shift condition compared to control or pH shift.

2.5. Evaluation of Temperature Shift Pre-Lyophilization

[00574] To evaluate temperature shift treatment pre-lyophilization and its effects on stability of the lyophilized material, Lyo6 was performed. This study simulated stress conditions potentially experienced during the fermentation process and was a measure to condition cells for the freezing process during lyophilization.

2.5.1. WP7-Lyo6.

[00575] Materials and Methods. The ADXS-HPV drug product was used for this study. No control group was included. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.5%, and the OD_{600} = 10.

[00576] Study Design. Once the material in the bioreactor reached the target OD_{600}, the required volume was harvested and the temperature shift was performed in an ice/salt/water bath, after which the material was stored at 2-8°C for 30 min. Then the material was processed further until formulated bulk was obtained. VCC was analyzed before and after the lyophilization run, which was performed similar to Lyo5, and at accelerated conditions for 1, 2, and 3 days (30°C). VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00577] Results. Accelerated results for ADXS-HPV were comparable to ADXS-HER2. One month stability was consistent with accelerated stability results. *See* **FIG. 9** (VCC) and **FIG. 26** (RM). The data demonstrate good stability under short-term accelerated conditions. The data at –20°C shows good stability at 12 months while the 2-8°C storage begins to show a loss in VCC after 12 months. The data show no clear trends in RM upon long-term storage at either –20°C or 2-8°C.

[00578] Conclusion. This study confirmed that the lyophilized ADXS-HPV construct produced results consistent with ADXS-HER2 and demonstrated the applicability of the lyophilization platform across constructs.

2.6. Evaluation of Reduced Primary Drying Shelf Temperature and Reduced Freezing Temperature.

[00579] To evaluate freezing temperature and primary drying shelf temperature and their effects on stability of the lyophilized material, Lyo7 was performed.

2.6.1. WP7-Lyo7.

[00580] Materials and Methods. The ADXS-HPV drug product was used for this study. No control group was included. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.5%, and the OD_{600} = 10. The stability conditions used were 2-8°C, –20°C, and accelerated conditions for 1, 2, and 3 days at 30°C.

[00581] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, the required volume was harvested and the temperature-shift was performed in an ice/salt/water bath, after which the material was stored at 2-8°C for 30 min. Then the material was processed further until formulated bulk was obtained. The lyophilization run was performed with freezing temperature decreased from –40°C to –45°C, and primary drying shelf temperature was decreased from –22°C to –30°C. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00582] **Results.** Significant losses were observed on accelerated stability with the decreased shelf temperature. *See* **FIG. 10** (VCC) and **FIG. 27** (RM). Decreasing shelf temperature during freezing did not improve stability of the lyophilized product.

2.7. Hold Time Study.

[00583] A hold time study was performed where the drug product (DP) was either lyophilized immediately after formulation or was frozen, thawed, and then lyophilized.

2.7.1. WP7-Lyo8.

[00584] **Materials and Methods.** The ADXS-HPV drug product was used for this study. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.5%, and the OD_{600} of the final formulated material = 10. The stability conditions used were 2-8°C, –20°C, and accelerated conditions for 1, 2, and 3 days at 30°C.

[00585] **Study Design.** In some groups (Part A), samples were lyophilized immediately. In other groups (Part B), samples were frozen at –80°C, thawed at 2-8°C overnight, and then lyophilized. VCC before lyo, after lyo, and at accelerated conditions for 1, 2 and 3 days (30°C) was analyzed. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00586] **Results.** Part A (continuous processing) demonstrated a better stability profile under accelerated conditions compared to Part B (frozen hold). *See* **FIG. 11** (VCC) and **FIG. 28** (RM).

2.8. Evaluation of Increased Primary Drying Shelf Temperature.

[00587] To evaluate an increased shelf temperature during primary drying and its effect on stability of the lyophilized material, Lyo9 was performed.

2.8.1. WP7-Lyo9.

[00588] Based on previous observations that a lower primary drying shelf temperature reduced the stability of the resulting drug product, the goal of WP7-Lyo9 was to evaluate the effect of a higher shelf temperature during primary drying on the stability of freshly (continuously processed) lyophilized drug substance ADXS11-001 (HPV). A shelf-temperature of -18°C during primary drying was evaluated. Lyophilized material was staged on stability at 2-8°C and –20°C. Further accelerated stability for 1, 2, and 3 days at 30°C/65% RH.

[00589] **Materials and Methods.** The ADXS-HPV drug product was used for this study. A shelf temperature of –18°C was evaluated. The temperature shift was performed similar to prior experiments. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.5%, and the OD_{600} = 10. The stability conditions used were 2-8°C, –20°C, and accelerated conditions for 1, 2, and 3 days at 30°C.

[00590] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, the required volume was harvested and the temperature-shift was performed in an ice/salt/water bath, after which the material was stored at 2-8°C for 30 min. Then the material was processed further until formulated bulk was obtained. The lyophilization run was performed with a primary drying shelf temperature of –18°C. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00591] **Results.** **FIG. 12** shows %VCC after lyophilization evaluated on stability at 30°C, –20°C and 2-8°C. **FIG. 29** shows residual moisture levels evaluated on stability at 30°C, –20°C and 2-8°. The data demonstrates that both –20°C and 2-8°C are stable up to 12 months. An improvement in accelerated stability was observed with increased shelf temperature. *See* **FIG. 12** (VCC) and **FIG. 29** (RM). In general, lyophilization of proteins at primary drying shelf temperatures high enough to cause the type of collapse seen in this study leads to reduced stability in the lyophilized cake. However, the trend for this whole bacteria formulation appears to be the opposite.

[00592] **Conclusion**. This study showed that increased primary drying shelf temperature resulted in improvement in accelerated and long-term stability for the lyophilized product.

2.9 Comparison of Plus/Minus Temperature Shift at Increased Primary Drying Temperature (–18°C).

[00593] To compare the minus [Part-A] / plus [Part-B] temperature shift at elevated primary drying temperature of Ts = –18°C, the Lyo10 stability study was performed.

2.9.1. WP7-Lyo10.

[00594] **Materials and Methods.** The ADXS-HPV drug product was used for this study. A shelf temperature of –18°C was evaluated for two groups of materials: (1) Part-A material - processed immediately after harvest (without temperature shift); and (2) Part-B material - temperature shift was performed.

[00595] The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 3.0%, and the OD_{600} = 10. The stability conditions used were 2-8°C, –20°C and accelerated conditions for 1, 2, and 3 days at 30°C.

[00596] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, the required volume for both Part-A and Part-B was harvested. Part-A was immediately processed until vial filling and for Part-B, temperature-shift was performed in an ice/salt/water bath, after which the material was stored at 2-8°C for 30 min. Then the Part-A and Part-B materials were processed further until formulated bulk was obtained. The lyophilization run was performed with a primary drying shelf temperature of –18°C and 2h secondary drying time to target a residual moisture of 3.0%. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00597] **Results.** With the increased shelf temperature, the results were comparable with and without the temperature shift. *See* **FIG. 13** (VCC) and **FIG. 30** (RM). The results confirmed the good stability profile previously observed for the increased shelf temperature.

2.10. Stability Study without Temperature Shift at Primary Drying Temperature of −18°C and Bioactivity Determination.

[00598] To confirm the results of Lyo10, a Lyo11 stability study was performed without temperature shift at elevated primary drying temperature of −18°C. Bioactivity of the lyophilized drug product was then compared to liquid frozen drug product.

2.10.1. WP7-Lyo11.

[00599] **Materials and Methods.** The ADXS-HPV drug product was used for this study. A shelf temperature of −18°C was evaluated without temperature shift. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 2.5-3.0%, and the OD_{600} = 10. The stability conditions used were 2-8°C, −20°C and accelerated conditions for 1, 2, and 3 days at 30°C.

[00600] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, the required volume was harvested and immediately processed until formulated bulk was obtained. The lyophilization run was performed with a primary drying shelf temperature of −18°C, and a residual moisture of 2.5-3.0% was targeted. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00601] **Results.** The data show that the temperature shift is not needed in order to achieve acceptable results when the shelf temperature is increased. *See* **FIG. 14** (VCC) and **FIG. 31** (RM). Lyophilized samples are stable out to 12 months for both 2-8°C and −20°C storage.

[00602] Advs11 frozen formulation (5271-15-01), lyo11 (5277 WP7 Lyo11) lyophilized formulation, and XFL7-tLLO-negative control strain were used to infect THP1 cells at MOI of 2 and 20. 10-mer peptide (YMLDLQPETT, SEQ ID NO: 100) was used as a positive control. Infected cells were then invocated for 20-24 hours, collected, and combined with T cells specific for the 10-mer peptide. After 18-24 hours, T cell IFNγ secretion was determined. A t low MOI, lyophilized formulation induced higher IFNγ production in the T cells. At higher MOI, lyophilized formulation showed similar induction of IFNγ production. The percent live for the lyophilized formulation was 95%. No loss in bioactivity was observed for the lyophilized product and for low MOI, bioactivity was increased. *See* **FIG. 39**.

2.11. Stability Study of Fresh vs. Frozen Material with Different Thawing

[00603] To confirm the results obtained from Lyo8, Lyo10, and Lyo11, a stability study Lyo12 was performed where a comparison was made between fresh and frozen material with the frozen material being thawed in different ways.

2.11.1. WP7-Lyo12.

[00604] **Materials and Methods.** The ADXS-HPV drug product was used for this study, and no temperature shift was performed. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 2.5-3.0%, and the OD_{600} = 10. The stability conditions used were 2-8°C, –20°C and accelerated conditions for 1, 2, and 3 days at 30°C. The groups tested were: (1) Group A: control, lyophilized directly; (2) Group B: frozen at <–70°C, thawed at 2-8°C; and (3) Group C: frozen at < –70°C, thawed at 37°C in water bath, then incubated for 4h at 37°C.

[00605] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, material was harvested and split into 3 aliquots for Parts A, B and C. Part A was immediately processed until formulated bulk was obtained and VCC analysis was performed before lyophilization. Part B and Part C materials were processed, aliquoted and frozen at <–70°C. Part B material was thawed at 2-8°C overnight and Part C material was thawed completely in a water bath at 37°C and was then incubated in the water bath at 37°C for 4h before lyophilization. Materials were then diluted to OD_{600} = 10, and processed for lyophilization. The lyophilization run was performed for a target residual moisture of 2.5-3.0%. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00606] **Results.** The data show that continually processed material has the better stability profile compare to frozen and thawed material. The data also demonstrate the drug substance may be stored prior to lyophilization. *See* **FIG. 15** (VCC) and **FIG. 32** (RM).

2.11.2. WP7-Lyo13.

[00607] **Materials and Methods.** The ADXS-HPV drug product was used for this study. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 2.5-3.0%, and the OD_{600} = 10. The stability conditions used were 2-8°C, –20°C and accelerated

conditions for 1, 2, and 3 days at 30°C. The groups tested were: (1) Group A: fresh, lyophilized directly; (2) Group B: stored at 2-8°C for 3 days.

[00608] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, material was harvested and split into 2 aliquots for Parts A and B. Part A was immediately processed until formulated bulk was obtained and VCC analysis was performed before lyophilization. Part B materials were processed, aliquoted and frozen at <–70°C. Part B material was stored at 2-8°C for 3 days before lyophilization. Materials were then diluted to $OD_{600} = 10$, and processed for lyophilization. The lyophilization run was performed for a target residual moisture of 2.5-3.0%. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00609] **Results.** The data show good results for both continually processed material (straight through processing) and material stored at 2-8°C for up to three days. *See* **FIG. 16** (VCC) and **FIG. 33** (RM). The data show that bulk drug substance (BDS) may be stored for three days at 2-8°C before processing and still achieve acceptable results post-lyophilization.

[00610] **Conclusion**. This study demonstrates that a 3 day hold of the drug substance at 2-8°C can still result in acceptable long-term stability of the lyophilized drug product allowing for adding flexibility during routine manufacturing.

2.12. Presentation of Product.

2.12.1. WP7-Lyo14.

[00611] **Materials and Methods.** The ADXS-HPV drug product was used for this study. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 2.5-3.0%, and the $OD_{600} = 10$. The stability conditions used were 2-8°C, –20°C and accelerated conditions for 1, 2, and 3 days at 30°C. The factors tested were 2R vials, 1×10^9 VCC, and 1.2 mL fill.

[00612] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, material was harvested and processed for lyophilization. The lyophilization run was performed for a target residual moisture of 2.5-3.0%. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization.

[00613] **Results.** The data show a decrease in viability under accelerated conditions but still within specifications. The data also indicate 2R vial presentation is suitable for use in lyophilization using the described compositions and methods. *See* **FIG. 17** (VCC) and **FIG. 34** (RM). The residual moisture was ~2% and below the target of 3-4%.

2.13 Batch Scale

[00614] **Materials and Methods.** The ADXS-HPV drug product was used for this study. The formulation was phosphate-based with 2.5% sucrose, the residual moisture target was 2.5-3.0%, and the OD_{600} = 10 with a target of 1×10^{10} CFU/mL. 2 mL ADXS-HPV drug product were added to each of about 1500 R6 vials. The stability conditions used were 2-8°C, –20°C and accelerated conditions for 1, 2, and 3 days at 30°C.

[00615] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, material was harvested and processed for lyophilization. The lyophilization run was performed for a target residual moisture of 2.5-3.0%. VCC was analyzed before and after lyophilization and at accelerated conditions. VCC titer was measured, expressed both as count and percent of count before lyophilization. Residual moisture (RM) was also determined.

[00616] **Results.** The described compositions and methods are suitable for use with scale batch lyophilization. Accelerated stability is consistent with development of scale batches. Batch scale demonstrated suitability for clinical supply of drug substance in 6R vial presentation. *See* **FIGS. 35-36** (VCC), **FIGS. 37-38** (RM), and **Table 12**. "H" and "C" in **FIG. 37** refer to hot and cold spots, respectively, within the lyophilizer.

[00617] **Table 12.**

Test	Test Method	Raw Data Reference	Result
Visual appearance	LAB-GEN-810 rev. 1.0	LAB-GEN-810-TR01: T17L004	20 vials with white to off white cake 20 vials with white to off white solution
pH	LAB-GEN-840 rev. 1.0	LAB-GEN-840-TR01: T17L007	pH: 6.77
Osmolality	LAB-GEN-800 rev. 1.0	LAB-GEN-800-TR01: T17L008	372 mOsm/kg
Extractable volume	LAB-GEN-820 Rev. 1.0	LAB-GEN-820-TR01: T17L005	1.92 mL per vial
CCIT	LAB-GEN-830 rev. 1.0	LAB-GEN-830-TR01: T17L006	No ingress of dye in 10 vials
Viable cell count	TM-SB-532 Rev. 2.0	TM-SB-532-TR01: T17L017	6.4×10^9 CFU/mL

2.14. Exemplary Materials and Methods.

[00618] **pH-Buffer Stock Solutions**. pH-buffer solutions were prepared as 4-fold stock solutions.

[00619] **Phosphate Buffer Stock Solution**. The phosphate buffer stock solution was prepared to resemble the current drug substance formulation as closely as possible, however without NaCl and KCl. It was prepared as a 4-fold stock: 0.8g of KH_2PO_4 (anhydrous) and 4.6g of Na_2HPO_4 (anhydrous) were dissolved in 800mL of WFI. pH was adjusted to 7.2 with 10% HCl. Solution was filled up to 1000mL of WFI and was filtered sterile with a 0.2 µm filter. The final formulated drug product would contain 0.2g/L KH_2PO_4 (anhydrous) and 1.15g/L of Na_2HPO_4 (anhydrous).

[00620] **Citrate Buffer Stock Solution**. A 40mM (=4-fold) stock solution of citric acid was prepared by dissolving 0.84g of citric acid *ad* 100mL WFI. A 40mM (=4-fold) stock solution of Na-citrate was prepared by dissolving 2.94g of Na-citrate *ad* 250mL WFI. A 40mM (=4-fold) stock solution of citrate buffer was prepared by adjusting the pH of the 40mM Na-citrate solution to pH=7.2 using the 40 mM citric acid solution. Solution was filtered sterile with a 0.2 µm filter.

[00621] **MOPS-Buffer Stock Solution.** A 40mM (=4-fold) stock solution of MOPS was prepared by dissolving 2.3125g MOPS in 200mL of WFI. pH was adjusted to pH=7.2 with 10% HCl. Solution was filled up *ad* 250mL WFI and was filtered sterile with a 0.2µm filter.

[00622] **40% w/v Sucrose Stock Solution.** 200g of sucrose were dissolved *ad* 500mL WFI. Solution was filtered sterile with a 0.2µm filter.

[00623] **40% w/v Trehalose Stock Solution.** 40g of trehalose were dissolved *ad* 100mL WFI. Solution was filtered sterile with a 0.2µm filter.

[00624] **20% w/v MSG Stock Solution.** 8g of L-Glutamic acid monosodium salt hydrate were dissolved *ad* 40mL WFI. Solution was filtered sterile with a 0.2µm filter.

[00625] **20% w/v rHSA Stock Solution.** 10g of lyophilized rHSA (Kerry, rAlbumin EG) were dissolved *ad* 50mL WFI. Solution was filtered sterile with a 0.2µm filter.

[00626] **Buffers for Resuspension of the Bacterial Pellet.** 1-fold buffers for resuspension of the bacterial pellet were prepared by mixing 140mL WFI, 10mL of 40% sucrose and 50mL of the respective 4-fold pH-buffer stock resulting in 1x pH buffer/2% sucrose.

[00627] **AA-Mix Stock Solution.** 200mL of a 4-fold amino acid stock solution were prepared that contained 144mM arginine, 228mM glutamine and 28mM isoleucine. *See, e.g.,* Paik *et.al.* (Biotechnol Prog. 2012 Nov-Dec; 28(6), herein incorporated by reference in its entirety for all purposes). Final amino acid concentrations in formulated drug substance were 36mM, 57mM and 7mM, respectively. It was prepared as follows: 5.17g of arginine (Mw=174.2 g/mol) were weighed and dissolved *ad* 50mL WFI. 48.52mL (representing 5.017g) of this solution were transferred into a 250mL bottle. 0.8g of isoleucine (Mw=131.17 g/mol) were weighed and dissolved *ad* 50mL WFI. 45.91mL of this solution (representing 0.735g) were transferred into the same 250mL bottle. 6.71g of glutamine (Mw=147.13 g/mol) were weighed and transferred directly into the 250mL bottle. Total volume was filled up to 160mL WFI and the pH was carefully adjusted to pH=7.2 by adding 2N NaOH under constant stirring. When all glutamine was dissolved and the pH was stable, volume was filled up to 200mL WFI. Solution was filtered sterile with a 0.2µm filter.

[00628] **Preparation of 2-Fold Excipient Stocks.** 2-fold excipient stocks were used for mixing 1:1 with the bacterial stock solution to obtain the final formulations with an OD_{600}~10 or OD_{600}~2.0. They were prepared by mixing appropriate volumes of 4-fold pH-buffer stock solutions and excipient stock solutions to obtain the desired concentrations.

[00629] **Cultivation of *Lm*.** 100mL of TSB (30g TSB / 1 kg WFI, 5g yeast extract / 1kg WFI, additional 7.5g glucose / 1 kg WFI giving a final concentration of glucose of 10g/1kg WFI) were pre-warmed in a 500mL baffled shake flask to 37°C. Medium was incubated overnight. On the next day, the medium was clear. One vial of the *Lm* RCB (5277-2015-01.01; VCC = 2.44×10^9 CFU/mL) was thawed at room temperature. Medium was inoculated with 900µL of vial content. *Lm* were incubated at 37°C, 110 rpm for 5h 15 minutes (P1). At this point the OD_{600} was 3.59. For P2, 5× 3L Fernbach bottles containing 500mL of TSB were inoculated with 2.5mL of P1, respectively. The cultures were incubated at 37°C, 110 rpm for 14h 50min. Cultures were pooled and OD_{600} was 5.96.

[00630] Alternatively, 50mL of TSB were pre-warmed in a 250mL baffled shake flask to 37°C. Medium was incubated overnight. On the next day, the medium was clear. One vial of the *Lm* RCB (5277-2015-01.01; VCC = 2.44×10^9 CFU/mL) was thawed at room temperature. Medium was inoculated with 600µL of vial content. *Lm* were incubated at 37°C, 110 rpm for 7h 55min (P1). At this point the OD_{600} was 4.78. For P2 3x 3L Fernbach bottles containing 500mL

of TSB were inoculated with 5.0mL of P1, respectively. The cultures were incubated at 37°C, 110rpm for 14h 30min. At this point the OD_{600} was 5.96.

[00631] **Concentration and Formulation of *Lm*.** For each pH-buffer (phosphate, citrate & MOPS) 520mL of P2 were centrifuged at 2,000g, 10 min, RT. Supernatant was discarded and the pellets were each resuspended in 155mL of 1x pH-buffer 2% sucrose, respectively and the OD_{600} values checked. Target OD_{600} was 20 after concentration: OD_{600} phosphate buffer = 19.2. OD_{600} citrate buffer = 18.6. OD_{600} MOPS buffer = 18.8. Final formulations were obtained by mixing equal volumes of concentrated bacteria with 2x-concentrated excipient stocks resulting in a formulation with an OD_{600}~ 10. Excipient concentrations are described elsewhere herein. After formulation a sample was taken for VCC analysis (before Lyo).

[00632] Alternatively, 1,000mL of P2 were centrifuged at 2,000g, 10min, RT. Supernatant was discarded and the pellets were each resuspended in 300mL of 1x phosphate buffer 2% sucrose. OD_{600} was 18.3 after resuspension. This stock was used for generation of the samples of OD_{600}~10. For generation of the samples of OD_{600}~2 this stock was diluted by a factor of 4.58 with 1x phosphate buffer 2% sucrose. OD_{600} was 4.12 after dilution. Final formulations were obtained by mixing equal volumes of concentrated bacteria with 2x concentrated excipient stocks resulting in formulations with an OD_{600}~10 or OD_{600}~2.

2.15. Summary.

[00633] In summary, the data show that accelerated conditions appear to be a good predictor of long-term stability and that storage of lyophilized drug product at 2-8°C and –20°C is possible. The data for ADVX-HPV are comparable to the data for ADXS-HER2, indicating that the data is predicted to be consistent across different *Lm* drug products. The data also indicate that higher RM is more desirable. For example, moistures below about 1% may not provide a stable *Lm* product, but moistures as high as 6-7% appear to be as stable as moistures around 3-4%. The data also indicate that the temperature shift improves stability, and that higher shelf temperature (primary drying step) improves stability. 2R and 6R vial presentation is suitable for use in lyophilization using the described compositions and methods. Bioassay shows good activity of lyophilized material compared to frozen liquid formulation. Batch scale is suitable clinical supply in a 6R presentation. In some embodiments, the drug product is presented in 6R

vials at 1×10^{10} CFU/mL. In some embodiments, the drug product is presented in 2R vials at 1×10^{9} CFU/mL.

[00634] Induction of a stress response through a temperature shift significantly improves the viability after lyophilization. In addition, despite phosphate buffers generally not being ideal buffers for lyophilization products, phosphate-based formulation had better performance compared to citrate- and MOPS-based buffers and required the smallest process change as they were closest to the current drug substance formulation. The best stability was seen in formulations including sucrose but no trehalose, MSG, or rHSA. Formulations containing sucrose-only or a mixture of sucrose + AA had better residual moisture compared to rHSA formulations, which were drier. Highest recoveries (and lowest variability) were observed at lowest sucrose concentration (about 2.5% w/v). Improvement in stability was observed with increased shelf temperature during the primary drying step (e.g., about 18°C) and with increased residual moisture levels (e.g., about 3.5%). For increased residual moisture levels such as 3.5% (which are higher than typical residual moisture levels for lyophilized products), secondary drying temperatures as low as 5°C may be feasible (e.g., between about 5°C and about 20°C).

Example 3. Reproduction and Further Optimization of Lyophilization Parameters for *Listeria monocytogenes*.

[00635] A series of experiments were performed with different test parameters to reproduce lyophilization cycles from Example 2 and to further optimize the formulation, the pre-conditioning of cells, and the lyophilization cycle.

3.1. Reproduce Previous Lyophilization Cycle as Basis of Comparison.

[00636] A series of experiments (WP2A) were performed with 6R vials and 2 mL fills using the same lyophilization cycle parameters as Lyo9 through Lyo13.

[00637] **Study Design.** Once the material in the bioreactor reached the target OD_{600}, material was harvested and processed for lyophilization. The lyophilization run was performed for a target residual moisture of 2.5-3.0%. VCC was analyzed before and after lyophilization and at accelerated condition (1, 2, and 3 days at 30°C). Residual moisture was also measured after lyophilization and at the 3 days accelerated condition. Micro-Flow Imaging (MFI) and Resonant Mass Measurement (RMM) were also performed before lyophilization, after lyophilization, and

at accelerated conditions for 1, 2, and 3 days (30°C). VCC titer was measured, expressed as both count and percent of count before lyophilization. Moisture and VCC data were compared to previous data from the same lyophilization cycle conditions.

[00638] **Results.** A decrease of the VCC (CFU/mL) to 80% was observed after lyophilization. *See* **FIG. 18**. The VCC was constant throughout storage for up to 72 hours at 30°C. Initial residual moistures (direct sample injection) averaged 2.4%, and no increase was observed over 72 hours at 30°C. The number of subvisible particles of samples before and after lyophilization was comparable as measured by MFI and RMM. The particle size distribution remained constant. The previous lyophilization cycle was successfully reproduced.

3.2. Study Residual Moisture Content as Function of Secondary Drying Shelf Temperature.

[00639] A series of experiments (WP2B) were performed to study residual moisture (RM) content as a function of secondary drying shelf temperature to predict secondary drying shelf temperatures that would result in the target of 3.5% RM.

[00640] **Study Design.** 6R vials with 2 mL fills and the same freezing and primary drying as in the WP2A experiments. Secondary drying was conducted in stages: (1) end of primary drying, stopper shelf (for RM information only); (2) ramp to 0°C, hold for 6 hr, stopper shelf; (3) ramp to 5°C, hold for 6 hr, stopper shelf; (4) ramp to 15°C, hold for 6 hr, stopper shelf. VCC was analyzed before and after lyophilization and at accelerated condition (1, 2, and 3 days at 30°C). Residual moisture was also measured after lyophilization and at the 3 days accelerated condition. MFI and RMM were also performed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C).

[00641] **Results.** The target RM of 3.5% can be obtained by using a secondary drying temperature between 5°C and 15°C (e.g., 12°C). *See* **FIG. 19**. The number of subvisible particles of samples at different sampling points was comparable as measured by MFI and RMM. Different SD temperatures had no influence on the subvisible particle level. Comparable particle levels were observed for WP2A samples. No significant change in VCC was observed from pre-lyophilization to post-lyophilization. A decrease of the VCC to 78%-85% of pre-lyophilization was observed after 72 hr at 30°C. There was no discernible trend of VCC with SD temperatures.

3.3. Evaluate Modified Freezing Steps.

[00642] A series of experiments (WP3) were performed to explore a modified freezing step with an extended hold time of vials at –4°C to allow all vials to equilibrate just above freezing temperature.

[00643] **Study Design.** 6R vials with 2 mL fills and the cycle conditions from the WP2A experiments were used with the following changes: (1) extend hold at –4°C from 30 min to approximately 1 hr 20 min; and (2) conduct secondary drying at 12°C to target 3.5% RM. VCC was analyzed before and after lyophilization and at accelerated condition (1, 2, and 3 days at 30°C). Residual moisture was also measured after lyophilization and at the 3 days accelerated condition. MFI and RMM were also performed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C).

[00644] **Results.** Primary drying (PD) of samples was competed after about 23 hours process time. For front vials, PD was already finished after 18 hours. For back vials, PD was finished after 20 hours. After PD, the vials were equilibrated for 83 min at –4°C. After lyophilization, samples were analyzed immediately (Tlyo) or stored at 30°C (Txxh). A decrease of the VCC to 80% was observed after lyophilization and to 70% after storage for up to 72 hr at 30°C (T30h). A RM of 2.3% was measured after lyophilization for the center vials instead of the expected 3.5%. The number of subvisible particles of the samples before and after lyophilization was comparable as measured by MFI. The particle size distribution remained constant. The enhanced freezing step may have changed the ice crystal formulation and thereby the drying behavior of the lyophilization cake, which may have influenced the cell viability and residual moisture. *See* **FIGS. 40A-B**.

3.4. Evaluate Modified Freezing Steps and Primary Drying Temperatures.

[00645] A two-factorial design study (WP4) is performed (freezing step and primary drying temperature).

[00646] **Study Design.** Fast shelf cooling from 5°C to –45°C during the freezing step is tested. Adjusted primary drying conditions are tested to reduce cake shrinkage/collapse. VCC is analyzed before and after lyophilization and at accelerated condition (1, 2, and 3 days at 30°C). Residual moisture is also measured after lyophilization and at the 3 days accelerated condition.

MFI and RMM are also performed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C).

3.5. Evaluate Thawing Procedures (37°C Thaw).

[00647] A series of experiments (WP6) were performed to evaluate a new thawing procedure for ADXS-HER2. The previous thawing procedure was to thaw formulated bulk material overnight at 2-8°C.

[00648] **Study Design.** 6R vials with 2 mL fills and the cycle conditions from the WP2B experiments were used. Formulated bulk material at an OD_{600} of 10 was thawed at 37°C. The cell pellet was thawed at 37°C and then diluted with formulation buffer to an OD_{600} of 10. VCC was analyzed before and after lyophilization and at accelerated condition (1, 2, and 3 days at 30°C). Residual moisture was also measured after lyophilization and at the 3 days accelerated condition. MFI and RMM were also performed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C). *See* **FIGS. 41A-B**.

[00649] **Results**. The process of WP2B was reproduced until SD temperature of 5°C. Primary drying (PD) of samples completed after ~ 25 h process time. Lyo process was comparable to WP2B. After lyophilization, samples were immediately analyzed (Tlyo) or stored at 30°C (Txxh). CFU/mL at Tliq were comparable between A (formulated bulk material (i.e., Drug Substance)) and B. (cell pellet (i.e., Drug Substance that has been highly concentrated to essentially remove all formulation buffer)). After lyophilization, a decrease to 70% and 80% VCC was observed. A further decrease to about 50 % VCC was observed after storage for 24 hours at 30°C, which was unchanged after 72Hr. at 30°C.

3.6. Evaluate Different Bacterial Target Concentrations (WP7).

[00650] Three different bacterial target concentrations were tested to determine the influence of the bacterial concentration on the cake appearance when the lyophilization cycle with an annealing step is used.

[00651] **Study Design**. Three different formulations with different OD600 values were prepared:

(a) OD 10: F1000: use of delivered BDS as provided,

(b) OD 2: F0200: 31.37 ml BDS + 118.63 ml formulation buffer, and

(c) OD 0.65: F0065: 10.20 ml BDS + 139.80 ml formulation buffer.

HER2 material was provided using the ADXS platform manufacturing process (*see* Example 7).

Parameter	Cell Pellet
Amount	800 mL (by volume
OD600 (raw material)	10.05
Cells/mL	1.69×10^{10}
Viability	98.45%

VCC was analyzed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2 and 3 days (30°C). Residual moisture was analyzed after lyophilization and 3 days accelerated condition. MFI and RMM were analyzed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C).

[00652] **Results**: Primary drying (PD) of samples was completed after ~40 h process time (indicated by both Pt_{100} sensor and pressure sensor readout). The process was comparable to WP7 cycle 1; only the SD temperature was changed from 5°C to 0°C to target a residual moisture content of ca. 3.5%. After lyophilization, samples were immediately analyzed (Tlyo) or stored at 30 °C or 2-8°C, respectively. A correlation between bacterial concentration and optical appearance of the final product was observed. The lower the bacterial concentration, the more cake shrinkage was observed. Reconstitution of the lyophilized cake was faster for F0065 and F0200 (~20s) than for F1000 (~100s). *See* **FIGS. 42A-B**.

[00653] After lyophilization, a decrease in VCC to about 60% was observed, independent of the bacterial concentration. Also after lyophilization, no difference in VCC was observed for front and center vials. A slightly higher VCC was even detected for front vials. After 24h at 30°C a further 10% decrease in VCC was observed for the two lower bacterial concentrations. Results were unchanged after 72 h. at 30°C. After 7 days at 2-8°C, a decrease in VCC of about 20% was observed. *See* **FIG. 43**.

[00654] Viable cell count (VCC) and viability appeared stable under accelerated conditions. *See* **FIGS. 44A-B**.

3.7. Evaluate 2R Vial Presentation (WP8).

[00655] 2R vial presentation at a target VCC of 1×10^9 wash was evaluated. In addition, stability of frozen and non-frozen BDS was compared.

[00656] **Study Design**. Formulated bulk material at a target VCC of 1×10^9 and 1×10^{10} plus about 30% to account for manufacturing losses was provided. VCC was analyzed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C). Residual moisture was analyzed after lyophilization and 3 days accelerated condition. MFI and RMM were analyzed before lyophilization, after lyophilization, and at accelerated conditions for 1, 2, and 3 days (30°C).

[00657] **Results**. Minimal losses due to lyophilization were observed. No significant changes observed in VCC or live/dead on accelerated stability were observed. % live at initial was higher relative to liquid-frozen formulation. *See* **FIGS. 45A-B**.

3.8 Large Scale Production (WP7).

[00658] In a large scale production the holding times of the BDS before starting a lyophilization run are longer compared to lyophilization runs during development in a pilot scale freeze dryer. The aim of this study is to evaluate whether this could have an influence on the product.

[00659] **Study Design.** Non-frozen liquid bulk drug substance (BDS) was provided by APC one day prior to the start of the freeze drying cycle. The liquid BDS was diluted to an OD_{600} value of 0.85 (target VCC of 1.3×10^9 CFU/ml) by using the delivered formulation buffer. The diluted material was stored at 2-8°C during the conduct of the holding time study. The four shelves were filled with BDS and loaded into the freeze-dryer at four different time points: 20 h (H20h), 8 h (H8h), 5 h (H5h), and 0 h (H0h) before starting the freeze-drying process.

[00660] **Results**. After lyophilization, a decrease in VCC was observed (relative to Tliq): 78% at H0h, 74% at H5h: 74%, 73% at H8h, and 65% at H20h. After storage for up to 72 h at 30°C a further decrease of around 10% was observed for H20h, H8h, and H5h. The VCC at T7 days after storage at 2-8°C was comparable to the VCC after lyophilization. *See* **FIGS. 46A**, **46B**, **47A**, and **47B**.

Example 4. Lyophilization of Drug Product and Long-Term Room Temperature Stability of the Lyophilized Drug Product.

4.1 ADXS11-001 Pilot Batch

4.1.1 Materials and Methods

[00661] Previous development experiments have all been performed using a small-scale development lyophilizer. Because scale up does not guarantee that the same dynamics of product temperature and ice content as those at the laboratory scale modifications to the lyophilization cycle may be needed for larger scale production. In order to evaluate potential scale up issues a pilot batch was manufactured for proof of concept and stability studies. The Drug Substance process is carried out within a single use closed system provided by rocking wave motion bioreactor technology. The Drug Substance manufacturing followed the ADXS platform manufacturing process. The platform consists of a single-use closed system of a product 20L culture bag for fermentation, a tangential flow filtration (TFF) manifold for concentration and buffer exchange and a container manifold for DS filling. The Drug Substance was held overnight at 2-8°C, diluted to the target OD_{600} with formulation buffer, filled into DIN 6R vials (2.0 mL) and lyophilized. The approximate batch size was 1500 vials.

[00662] Table 13. Primary Packaging Materials used for Pilot Batch.

Primary Packing Material	Specifications	Supplier
6*R* Glass vials	Rofa, 6R	HAL Allergy, MW005-002
Lyo stoppers Ready to sterilize (steam sterilization)	West, 7000-5780	Adelphi, FDW20RTS
Crimp cap, flip-off seals	West, 5921-2032	Adelphi, FOT20W

The stoppers were delivered ready-to-process and were not dried before use.

4.1.2 Study Design

[00663] The lyophilization process was conducted in a Martin Christ Epsilon 2-12D pilot-scale lyophilizer. Because this lyophilizer uses a Pirani gauge as the controlling pressure sensor instead of an MKS sensor used in the laboratory-scale lyophilizer, a pressure set point for the Pirani gauge had to be selected. Based on a review of previous lyophilization cycles where the Pirani gauge pressure was measured (but not used for control), a Pirani pressure of 0.163 mbar was found to be equivalent to a MKS pressure of 0.090 mbar during the main portion of primary drying. Since Pirani gauge pressure is dependent upon the composition of the gas phase, as the

partial pressure of water decreases towards the end of primary drying, the Pirani pressure readings approach the MKS pressure readings.

[00664] Table 14. Lyophilization Parameters for ADXS11-001 Pilot Batch

Step	Phase		Item	Duration [hh:mm]	Temp, T_s of Shelves [°C]	Vacuum [mbar]
1	Preparation		Warm up of lyo, placement of temperature and Rx sensors	NA	4	off
2	Load		Loading of Shelves	NA	4	off
3	Ramp	Freezing	Ramp samples to −4°C (0.4°C/min)	00:20	-4	off
4	Hold		Hold/anneal samples to −4°C	00:30	-4	off
5	Ramp		Freeze ramp to −45°C (0.45°C/min)	01:30	-45	off
6	Hold		Hold at −45°C, temperature equilibration across load	01:30	-45	off
7	Hold		Hold and preparation of vacuum pump	00:30	-	-
8	Ramp	Primary Drying	Vacuum[1]	00:01	-45	0.160***
9	Hold		Vacuum[1]	00:15	-45	0.160
10	Ramp		Heating ramp (1.0°C/min)	00:27	-18	0.160
11	Hold		Stable shelf temp:primary drying	>tbc[2]	-18	0.160
12	Ramp	Secondary Drying	Heating ramp (0.2°C/min)	03:10	20	0.160
13	Hold		Stable shelf temp:secondary drying	02:00	20	0.160
14	Ramp- Hold		Ramp to 10°C	00:10	10	0.160
15	Hold		Hold at 10°C until unloading	NA	10	1.0[3]
15a			Ventilation and manual vial closure	NA	10	500mbar

[1]Pirani vacuum sensor is process controlling (***0.163 was not programmable)

[2]End of primary drying is defined here 14 hours after T_P P100 probe for the cold spot(s) have crossed the T_s set point of -18°C

[3]Only done to avoid vacuum pull after the cycle has finished during the night

4.1.3 Results and Discussion

[00665] Table 15. OD_{600} and VCC for In-Process Samples

Process Step	OD_{600}	VCC
WAVE Harvest	7.9	1.76×10^{10}
Pre-Formulation	17	3.09×10^{10}
Final Bulk Formulation Post Hold/Final Formulation	10.2	1.27×10^{10}
Final Formulated Bulk (before Lyo	10.2	1.76×10^{10}

[00666] Preliminarily mapping of the lyophilizer was performed by determining the VCC (plate method) for hot (H) and cold (C) spots per shelf within the lyophilizer. The data are presented in **FIG. 48** and **Table 16**.

[00667] Table 16. VCC and Residual Moisture Data from Hot and Cold Spots within the lyophilizer for the Pilot Batch

Location	Sample	VCC (CFU/mL)	Residual Moisture
Hot	1	7.00E+09	2.57
	2	7.60E+09	2.04
	3	6.58E+09	2.39
	4	6.94E+09	2.37
	5	7.28E+09	2.16
Cold	1	9.78E+09	2.45
	2	1.02E+10	2.53
	3	1.01E+10	2.81
	4	1.03E+10	2.61
	5	1.12E+10	2.42

[00668] Summary statistics for the VCC data from the Hot and Cold spots within the lyophilizer demonstrate that the mean VCC for the Hot spots is 7.08E+09 CFU/mL and the mean for the Cold spots is 1.032E+10 CFU/mL. Hot spots and cold spots in the lyophilizer are determined based on temperatures from probes within the lyophilizer. Hot spots tend to be on the edges of the lyophilizer, and cold spots tend to be in the center of the lyophilizer. The sample numbers correspond to the shelf within the lyophilizer and show little variation with the H or C locations. Refer to the CV column in **Table 17**.

[00669] Table 17. Statistics Summary

Variable	Location	N	Mean	StDev	Coef Var (CV)
VCC (CFU/mL)	Cold	5	10316000000	531300292	5.15
C	Hot	5	7080000000	382883794	5.41

[00670] The data show a distinction in VCC and RM between the hot and cold Spots within the lyophilizer. It is not known if the difference in VCC is necessarily due to the difference in RM, or whether they are both functions of some other characteristic of the lyophilization cake.

[00671] Release and stability analysis was performed by Eurofins using validated methods. Vials are reconstituted with 2 mL or normal saline prior to analysis. The release and stability data are provided in **Table 18**.

[00672] Table 18. Release and Stability Testing for ADXS11-001 Pilot Batch (Lot# 5329PD-17-01)

Test	Test method		Specifications	Results								
				Release testing	Accelerated stability			T1		T3		T6	
					Day 1	Day 2	Day 3	5°C	−20°C	5°C	−20°C	5°C	−20°C
Visual appearance	LAB-GEN-810	Cake	White/off-white to slightly yellow powder									Intact cake	Intact cake
		Color - Reconstituted Solution	White to off-white suspension	20 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color	12 vials with white to off white color
		Particles-Reconstituted Solution	Essentially free of foreign particles	20 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present	12 vials with no particles present
pH	LAB-GEN-840		6.0-8.0	6.77	6.79	6.80	6.79	6.80	6.81	6.79	6.79	6.87	6.86
Osmolality	LAB-GEN-800		250-450 mOsm/kg	372	-	-	-	-	-	-	-	-	-
Extractable volume	LAB-GEN-820		>1mL/vial	1.92 mL	-	-	-	-	-	-	-	-	-
CCIT	LAB-GEN-830		No Dye Ingress	No ingress of dye in 10 vials	-	-	-	-	-	-	-	-	-

Reconstitution time	LAB-GEN-880		Report Result (seconds)	56	24	28	27	20	22	21	21	36	29
Viable cell count	TM-SG-110		5×10^9 – 5×10^{10} CFU/mL	1.12×10^{10}	1.16×10^{10}	1.09×10^{10}	1.10×10^{10}	1.24×10^{10}	1.22×10^{10}	1.15×10^{10}	1.11×10^{10}	1.15×10^{10}	1.21×10^{10}
% of viable cells	TM-SG-110		>60%	86%	89%	83%	87%	88%	87%	88%	88%	88%	89%
Endotoxin	TM-SG-101		EU/mL	<10 EU/mL	-	-	-	-	-	-	-	-	-
Monosepsis	TM-SB-534	TYMC < 10 CFU/mL		< 1 CFU/L	-	-	-	-	-	-	-	-	-
		TYMC: *Candida albicans*	Absent in 1 mL	Absent in 1 mL	-	-	-	-	-	-	-	-	-
		Absent in 1 mL •*Escherichia coli* • *Bacillus subtilis* • *Pseudo-monas aeruginosa* • *Staphylo-coccus aureus* • *Clostridia sp.*	Absent in 1 mL	Absent in 1 mL	-	-	-	-	-	-	-	-	-
		Absent in 10 mL • Salmonella sp.	Absent in 10 mL	Absent in 10 mL	-	-	-	-	-	-	-	-	-
		Single Species (*L. monocytogenes*)	Pure culture	Pure culture	-	-	-	-	-	-	-	-	-

4.2.1 Accelerated Stability

[00673] Early development data demonstrated that accelerated stability at 30°C may be predictive of long-term stability trends. The batch was stored at 30°C and evaluated for up to 63 days to determine how long the product was stable for under accelerated conditions (**FIGS. 49-53** and **Table 19**).

[00674] Table 19. VCC and % Live for ADXS11-001, Lot# 5329PD-17-01 Stored at 30°C

Day	Specification	% Live	VCC (cells/mL)
0	≥60%	86%	1.12×10^{10}
1		83%	1.32×10^{10}
3		86%	1.35×10^{10}
7		89%	1.43×10^{10}
14		91%	1.09×10^{10}
21		92%	1.51×10^{10}
23		88%	1.46×10^{10}
28		91%	1.01×10^{10}
35		90%	1.11×10^{10}
42		90%	1.08×10^{10}

4.2.2 *In Vivo* Testing

[00675] ADXS11-001 (AXAL) is a live attenuated Listeria monocytogenes-listeriolysin O (Lm-LLO) immunotherapy that is under clinical development for the treatment of human papilloma virus (HPV)-associated cancers. ADXS11-001 is bioengineered to secrete an antigen-adjuvant fusion protein consisting of a truncated fragment of listeriolysin O (tLLO) fused to the full length E7 protein of HPV 16 (tLLO-E7). The proposed mechanism of action of the Lm-based immunotherapy is to stimulate both the innate and adaptive immune systems in order to initiate a coordinated anti-tumor response culminating in the de novo generation of tumor antigen-specific T cells that are capable of infiltrating and destroying the tumor. In order to confirm that the bioactivity of the product is not adversely impacted by lyophilization tumor-bearing mice immunized with ADXS11-001 generate CD4+ and CD8+ T cells specific to HPV16-E7 and HPV16-E6.

[00676] The abilities of lyophilized AXAL and clinical AXAL to control tumors and to prolong animal survival were evaluated and compared in TC-1 tumor-bearing mice. Adult female C57BL/6 mice were injected subcutaneously in the right flank with $1x10^5$ TC-1 tumor cells and then immunized on Days 8, 15, and 22 after tumor implantation by IP injection with PBS or with various doses ($5x10^7$ CFU, $1x10^8$ CFU, $2x10^8$ CFU) of lyophilized AXAL or

clinical AXAL (*see* **FIG. 54**). Tumor growth and the general health of the mice were monitored for 62 days after tumor implantation. Mice were euthanized if tumor volume exceeded 2000 mm^3.

[00677] In mice treated with PBS, tumor volume continued to increase, and no animals survived past Day 30 (**FIGS. 55-56**). In comparison, all doses of lyophilized AXAL and clinical AXAL significantly inhibited tumor growth and prolonged animal survival (**FIGS. 55-56**). Notably, for each dose, the tumor growth curves and survival curves for lyophilized AXAL and clinical AXAL were similar.

[00678] As shown in **FIG. 55**, tumor-bearing mice were treated on day 8 post-tumor implantation with PBS or with 3 different doses of lyophilized AXAL or clinical AXAL and at 7-day intervals thereafter for a total of 3 doses. Tumor volume was measured twice a week. Tumor growth curves for each dose group are shown. ****P< .0001. NS, not significant.

[00679] As shown in **FIG. 56**, tumor-bearing mice were treated on day 8 post-tumor implantation with PBS or with 3 different doses of lyophilized AXAL or clinical AXAL and at 7-day intervals thereafter for a total of 3 doses. Tumor growth and the general health of the mice were monitored for 62 days after tumor implantation. Mice were euthanized if tumor volume exceeded 2000 mm^3. Survival curves for each dose group are shown. **P< .01. NS, not significant.

[00680] No significant differences were observed between lyophilized AXAL and clinical AXAL in their abilities to control tumor growth and to prolong animal survival in TC-1 tumor-bearing mice. These data indicate that the lyophilization process does not affect the antitumor activities of AXAL.

4.3 Conclusion

[00681] The pilot batch successfully demonstrated the application of the ADXS DS platform manufacturing process to support a lyophilized Drug Product. The % live and VCC on accelerated stability is consistent with previous development studies.

Example 5. Ability to Freeze/Thaw the Drug Substance and Obtain Comparable Results to Continuously Processed Material

5.1 WP7, Cycle 3

[00682] Different storage conditions of Drug Substance (frozen *vs.* non-frozen 2-8°C) were evaluated to see if the improvements in the lyophilization cycle result in improved viability post lyophilization for DS that has been through a single freeze-thaw.

5.2 Materials and Methods

[00683] Frozen (A) and non-frozen liquid (B) BDS were provided by APC (Dublin, Ireland) one day prior to the freeze-drying cycle. Approximately 800 mL of both 2-8°C and Frozen DS was formulated to an approximately OD_{600} of 14 in 1L LDPE bags. The frozen material was thawed in a water bath at 37°C until the material contained no long contained ice crystals (thawing time: 2.5h). The two Drug Substances (A and B) were diluted to two different OD_{600} values by using the formulation buffer (prepared at Coriolis). **Table 20** gives an overview of the prepared formulations and the target OD_{600} values.

[00684] Table 20. Formulations and Target OD_{600} values

Formulation	BDS	OD_{600} Value
A0085	Frozen Drug Substance	0.85
A1300	Frozen Drug Substance	13
B0085	Non-Frozen Drug Substance	0.85
B1300	Non-Frozen Drug Substance	13

[00685] The measured OD_{600} values of the Drug Substance as well as of the dilutions are shown in **Table 21**. The dilution scheme for the prepared formulation is given in **Table 22**.

[00686] Table 21. Measured OD_{600} Values of the Delivered Material.

BDS (1000 mL, by volume)	OD_{600} values	
	A: frozen material	B: non-frozen material
According to shipping documents	13.8	13.8
Measured at Coriolis, n=3	12.14 ± 0.12	11.33 ± 0.18
Formulations	**OD_{600} Values**	
A0085	0.86 ± 0.00	
A1300	12.14 ± 0.12	
B0085	0.87 ± 0.00	
B1300	11.33 ± 0.18	

[00687] Table 22. Dilution Scheme for the Three Formulations – F1000, F0200, and F0065.

Formulation	DS	Formulation Buffer
A0085	14.00 mL	186.00 mL
A1300	200.00 mL	-
B0085	15.01 mL	184.99 mL
B1300	200.00 mL	-

5.3 Study Design

[00688] Freezing during lyophilization was performed without an annealing step or hold at 4°C, since no positive effect on the cake appearance was observed with those steps in previous experiments. The vials were immediately frozen to –45°C without a holding or annealing step. The secondary drying time was prolonged to 5 hours to obtain a more homogeneous batch and to reach the target residual moisture content (RM) of 3.5%. RM, VCC, MFI, RMM, reconstitution time, classification of the appearance of the lyophilized products, and determination of the water loss by weighing was performed after lyophilization. VCC, MFI, RMM were analyzed at 30°C for 24 and 72h. Additionally, VCC was analyzed for samples at 2-8°C for 7 days.

[00689] Table 23. Lyophilization Cycle Parameters for WP7, Cycle3

Step	Time	Temperature [°C]	Pressure* [mbar]	Total Time [h]	Ramp [K/min]
loading	00:00**	4	1000	0.0	
freezing	00:49	-45	1000	0.8	1.00
freezing	02:00	-45	1000	2.8	
Primary drying	00:30	-45	0.09	3.3	
Primary drying	00:15	-45	0.09	3.6	
Primary drying	00:27	-18	0.09	4.0	1.00
Primary drying	32:00:00	-18	0.09	36.0	
Secondary drying	01:30	0	0.09	37.5	0.20
Secondary drying hold	05:00	0	0.09	42.5	
aeration	00:30	0	500	43.0	
stoppering	00:01**	0	500	43.0	
storage	00:30**	5	1000	43.1	0.50

*Pirani gauge controlling

5.4 Results and Discussion

[00690] Lyophilization was performed using an Epsilon 2-12D pilot scale freeze-dryer (Martin Christ, Osterode, Germany). During the freeze-drying process pressure (by Pirani and MKS), product temperature, shelf temperature, and ice condenser temperature were monitored. Center and front vials were monitored by PT_{100} sensors.

[00691] No annealing step was included during freezing and the secondary drying step at 0°C was set to 5h. The primary drying of all samples was completed after about 34h process time, as indicated by PT_{100} sensors and pressure sensor readout. For front vials the primary drying was already finished after 18h process time as indicated by PT_{100} sensors. The drying of the samples was independent of the bacterial concentration.

[00692] For the freezing step, the samples were immediately frozen to –45°C without a holding or annealing step. The front vials did not reach –45°C before ramping to the PD temperature, according to the PT_{100} sensors.

[00693] A video was recorded from the lyophilization process to determine when the shrinkage takes place. In the video, samples from all four formulations (A0085, A1300, B0085 and B1300) were visible and the shrinkage seems to occur during primary drying. The drying behavior of the center vials might be a slightly different as primary drying was finished earlier for front vials than for center vials.

5.5 Optical Evaluation of the Freeze-Dried Product

[00694] The optical appearance of the lyophilized cakes was documented for ten center vials of each formulation after lyophilization. The overall optical appearance of the lyophilization cakes of the four formulations was good. All cakes were compact and had no full contact to the glass vial. For the higher concentrated formulations (A1300 and B1300) the shrinkage in cake height and from the vial wall and bottom was comparable to that of samples of the previous cycle with similar OD_{600} values (F1000). The same is true for the lower concentrated samples (A0085 and B0085). A similar lyophilization cake was observed as for the lower concentrated formulations of Cycle 2 (F0065). The lyophilization cake had a similar optical appearance as the lyophilized placebo. As with cycle 2, a correlation between bacterial concentration and optical appearance of the final product was observed.

5.6 Determination of the Cake Weight

[00695] The cake weight and the water loss were determined during cycle 3. The weight of five vials per formulation was gravimetrically determined. Based on the weight of the empty vial, the vial after filling and after lyophilization, the cake weight and the water loss were calculated (**Table 24**). The lyophilization cake of the lower concentrated formulations weighed

30 mg and that of the higher concentrated formulations 40 mg. After lyophilization, a water loss of 1.17-1.18 g was determined. Therefore, a reconstitution volume of 1.2 mL is suitable to obtain the same bacterial concentration as prior to lyophilization.

[00696] Table 24. Determination of the Cake Weight and the Water Loss.

Sample		Empty Vial (g)	Filled Vial (g)	Vial after Lyophilization (g)	Cake Weight (g)	Water Loss (g)	Water Loss (%)
A1300	1	5.20	6.41	5.24	0.04	1.17	82
	2	5.27	6.48	5.30	0.04	1.18	82
	3	5.23	6.44	5.27	0.04	1.18	82
	4	5.16	6.37	5.20	0.04	1.17	82
	5	5.17	6.38	5.21	0.04	1.17	82
A0085	1	5.18	6.39	5.21	0.03	1.18	82
	2	5.21	6.41	5.24	0.03	1.17	82
	3	5.15	6.36	5.18	0.03	1.18	81
	4	5.22	6.43	5.25	0.03	1.18	82
	5	5.21	6.41	5.24	0.03	1.18	82
B1300	1	5.28	6.49	5.31	0.04	1.17	82
	2	5.27	6.48	5.31	0.04	1.17	82
	3	5.21	6.42	5.25	0.04	1.17	82
	4	5.18	6.38	5.21	0.04	1.17	82
	5	5.21	6.42	5.24	0.04	1.17	82
B0085	1	5.26	6.46	5.29	0.03	1.18	82
	2	5.25	6.47	5.29	0.03	1.18	82
	3	5.26	6.47	5.29	0.03	1.18	82
	4	5.23	6.44	5.26	0.03	1.18	82
	5	5.27	6.48	5.30	0.03	1.18	82

5.7 Reconstitution Time

[00697] The reconstitution time was measured for two samples per formulation and compared to the results of cycle 1 and cycle 2 (**FIG. 57** and **Table 25**). The reconstitution time of the higher concentrated formulations (A1300 and B1300) was longer than for the lower concentrated formulations (A0085 and B0085). The reconstitution times were, in general, shorter than for the previous cycles of WP7. A direct freezing of the samples without including an annealing step seems to shorten the reconstitution times.

[00698] Table 25. Overview of the Measured Reconstitution Times of WP7 Cycle 1 to Cycle 3.

Reconstitution time					
Sample	Time (sec)			Foam	LP
		Mean	SD		
Cycle 1_SD start_1	90	95	5	-	yes
Cycle 1_SD start_2	100			-	yes
Cycle 1_SD 2h_1	75	73	2	-	yes
Cycle 1_SD 2h_2	71			-	no
Cycle 1_SD end_1	77	69	8.5	-	no
Cycle 1_SD end_2	60			-	no
Cycle 2 F0065_2	24	22	2	-	no
Cycle 2 F0065_3	20			-	no
Cycle 2 F0200_1	21	21	0.5	-	no
Cycle 2 F0200_2	20			-	yes
Cycle 2 F1000_1	107	104	3.5	-	no
Cycle 2 F1000_2	100			-	yes
Cycle 3 A0085_1	13	12	1	-	no
Cycle 3 A0085_2	11			-	no
Cycle 3 A1300_1	47	53	6	-	no
Cycle 3 A1300_2	59			-	yes
Cycle 3 B0085_1	13	14	1	-	no
Cycle 3 B0085_2	15			-	no
Cycle 3 B1300_1	72	63	9.5	-	no
Cycle 3 B1300_2	53			-	no

Score Foam: 0 – no foam, 1 slight foam, 2 – moderate foam, 3 – strong foam, 4 – strong and steady foam.

LP – low pressure.

5.8 Micro-Flow Imaging (MFI)

[00699] The number of subvisible particles was analyzed by MFI to determine whether the raw material (frozen or non-frozen) or the bacterial concentration has an influence on particle formation as well as on the size distribution of the particles. Subvisible particles are particulate matter that is not observable to the naked eye. Particulate matter in injections and parenteral infusions consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions. There are regulatory limits on the number of subvisible particles allowed in parenteral infusions. Samples were analyzed before lyophilization (Tliq), after lyophilization (Tlyo), and after storage for 24 hours and 72 hours at 30°C (T24h and T72h). The results are shown in **FIGS. 58A-D**. The number of subvisible particles was unchanged for the frozen material (A0085 and A1300) before and after lyophilization as well as after storage at 30°C for up to 72h. For the non-frozen material, more particles were detected before lyophilization. Similar results were obtained in other experiments where non-frozen material

was also used. The number of subvisible particles after lyophilization was comparable to the results of the frozen material.

5.9 Resonant Mass Measurement (Archimedes)

[00700] Results for RMM analysis of ADXS-HER2 samples regarding their content of negatively and positively buoyant particles are presented in **FIGS. 59A-D**.

[00701] The results for the frozen and the non-frozen material were similar. Comparison of cumulative negatively buoyant particle counts for all time points and all storage conditions for bin size ≥0.3 µm. Note that particle counts below 300,000 particles per mL (LoQ), are given for information only (data not dilution-corrected, A0085 and B0085: 200-fold dilution, A1300 and B1300: 5,000-fold dilution).

[00702] After lyophilization a smaller second particle population appeared at around 300 nm. The particle distribution was comparable for all formulations throughout all analyzed time points. Comparison of the differential particle counts of negatively buoyant particles. Values below 0.3 µm size bins are given for information only (data not dilution-corrected, A0085 and B0085: 200-fold dilution, A1300 and B1300: 5,000-fold dilution).

[00703] Because of the different bacterial concentrations of the formulations, different dilutions had to be prepared. A0085 and B0085 were 200-fold diluted and A1300 and B1300 were diluted 5,000-fold. The cumulative counts are not corrected for the individual dilution, in order not to overestimate the multiplication error inherent to the measurement. Furthermore, since the Limit of Quantitation (LoQ) of the method is approximately 300,000 particles per mL, a dilution correction would elevate low particle counts for some measurements above the LoQ, which would otherwise not have been considered and might therefore not reflect the actual experimental conditions.

[00704] The number of submicron particles was unchanged for all four formulations before and after lyophilization (**FIGS. 59A-D**). The results for the frozen and the non-frozen material were similar. Before lyophilization one main particle population was detected in the size range of 600 – 700 nm (**FIGS. 60A-D**). After lyophilization, a smaller second particle population appeared at around 300 nm. The particle distribution was comparable for all formulations throughout all analyzed time points.

5.10 Karl Fischer Titration

[00705] The RM at Tlyo was analyzed by direct injection (**FIG. 61** and **Table 26**). A RM of about 3% was reached for the higher concentrated samples (A1300 and B1300) and a RM of about 3.5% for the lower concentrated samples (A0085 and B0085). The relative standard deviation of the five analyzed vials per formulation was lower than for cycle 2, which is most likely due to the extended SD time. The results of the frozen and non-frozen material were comparable.

[00706] Table 26. Overview of the KF Results Measured by Direct Injection for WP7 Cycle 3.

Sample	RMC [%]			
	A0085	A1300	B0085	B1300
Vial 1	3.4	3.1	3.3	3.3
Vial 2	3.1	3.0	3.5	3.4
Vial 3	3.3	2.8	3.5	3.2
Vial 4	3.7	3.2	4.1	2.7
Vial 5	3.2	2.8	3.9	2.5
Mean	3.4	3.0	3.7	3.0
SD	0.2	0.2	0.3	0.3

5.11 VCC Assay

[00707] The concentration of viable bacteria (VCC, expressed as CFU/mL) was analyzed at Tliq, after lyophilization at Tlyo, after storage for 24 h and 72 h at 30°C (T24h and T72h) and after storage for 7 days at 2-8°C (T7days) (**FIG. 62** and **Table 27**). After lyophilization, a decrease in VCC to about 60% (relative to Tliq) for the lower bacterial concentration and to 70-78% for the higher bacterial concentration was observed. A further decrease of about 10% was observed for the lower bacterial concentration and of 20% for the higher bacterial concentration after an incubation time of 72 h at 30°C. The VCC results after storage for 7 days at 2-8°C were comparable to the values after lyophilization. The results for the CFU/mL were, as in WP2, below the target values post-lyophilization:

- A1300 (target OD_{600} of 13): 1E+10 CFU/mL
- A0085 (target OD_{600} of 0.85): 1E+09 CFU/mL
- B1300 (target OD_{600} of 13): 1E+10 CFU/mL
- B0085 (target OD_{600} of 0.85): 1E+09 CFU/mL

APC (Ireland, Dublin) performed flow cytometry again with samples of each formulation. The VCC and % live results are presented in **FIG. 62** and **Table 27**.

[00708] Table 27. Overview of the CFU/mL and the Relative Viability.

Sample		CFU/mL	SD	%
A0085	Tliq	5.67E+08	1.21E+08	100
	Tlyo	3.17E+08	1.56E+07	56
	T24h	2.48E+08	2.00E+07	44
	T72h	2.43E+08	1.26E+07	43
	T7days	2.94E+08	2.06E+07	52
A1300	Tliq	5.63E+09	1.43E+09	100
	Tlyo	4.40E+09	3.83E+08	78
	T24h	3.40E+09	2.08E+08	60
	T72h	3.39E+09	1.55E+08	60
	T7days	3.61E+09	2.56E+08	64
B0085	Tliq	5.03E+08	8.06E+07	100
	Tlyo	3.41E+08	2.19E+07	68
	T24h	2.84E+08	1.89E+07	56
	T72h	2.69E+08	2.24E+07	54
	T7days	3.15E+08	1.45E+07	63
B1300	Tliq	6.23E+09	6.94E+08	100
	Tlyo	4.37E+09	3.14E+08	70
	T24h	3.24E+09	2.76E+08	52
	T72h	3.20E+09	1.33E+08	51
	T7days	3.91E+09	1.72E+08	63

[00709] FIGS. 63A-B show the VCC and % live after Cycle 3 and on accelerated stability. Minimal losses were observed due to lyophilization. No changes were observed in VCC or % live on accelerated stability. % live at initial is higher relative to the liquid-frozen formulation, which supports the further development of a lyophilized formulation. DS that had undergone a freeze/thaw demonstrated good accelerated stability with no decrease observed in either VCC or % live. There is a slight offset in % live observed between fresh and frozen material at the lower VCC levels which is consistent with prior observations that increased VCC is associated with better recoveries post-lyophilization.

[00710] Table 28. VCC and % Live for WP7, Cycle 4 Stored at 30°C

Sample			CFU/mL	% Live
2-8°C Stored DS	F1300	PreLyo	1.86E+10	96.6
		T=0h	1.55E+10	94.7
		T=24h	1.53E+10	94.7
		T=48h	1.62E+10	94.5
		T=72h	1.53E+10	94.3
	F0085	PreLyo	1.38E+09	93.3
		T=0h	1.40E+09	95.7
		T=24h	1.32E+09	95.7
		T=48h	1.37E+09	94.1
		T=72h	1.33E+09	94.2
Frozen DS	F1300	PreLyo	1.70E+10	96.1
		T=0h	1.52E+10	93.4
		T=24h	1.46E+10	93.9
		T=48h	1.40E+10	93.7
		T=72h	1.48E+10	93.4
	F0085	PreLyo	1.28E+09	90.1
		T=0h	1.24E+09	94.0
		T=24h	1.19E+09	92.9
		T=48h	1.19E+09	90.9
		T=72h	1.18E+09	90.8

5.12 Conclusion

[00711] Lyophilization runs with non-frozen and frozen DS were performed. Two target bacterial concentrations were tested. No annealing step or hold was included during freezing, as there were no advantages during the previous cycles.

[00712] The optical appearance of the lyophilization cake of all formulations (two different bacterial concentrations, frozen and unfrozen raw material) was good. The shrinkage of the lyophilization cake appeared to be dependent on the bacterial concentration. Less shrinkage was observed for a higher bacterial concentration.

[00713] Reconstitution time was dependent on the bacterial concentration and on the freezing step. Longer reconstitution times were observed for a higher bacterial concentration. Shorter reconstitution times were observed in these experiments, which did not have an annealing step during freezing, compared to experiments having an annealing step during freezing (data not shown).

[00714] The number of subvisible and submicron particles was mainly unchanged after lyophilization and storage at 30°C (analyzed by RMM and MFI). The number of particles of the

non-frozen material was higher before lyophilization. After lyophilization, a small particle population with a size of about 300 nm was detected (analyzed by RMM).

[00715] A decrease of the plate based VCC to about 60-70% (relative to Tliq) was observed after lyophilization for the lowest bacterial concentration. A higher VCC (70-78% relative to Tliq) was observed for the two higher bacterial concentrations. A further decrease of 10-20% was observed after storage for up to 72h at 30°C. Storage of the DS in a 1L LDPE bag with thawing at 37°C demonstrated comparable VCC and % live results on accelerated stability to the DS that was continuously processed.

[00716] Flow Cytometry Analysis for VCC and % live demonstrated good stability for both the frozen and fresh DS. There was a slight offset for the lower VCC level between the fresh and frozen DS.

[00717] A RM of about 3% (higher bacterial concentration) and 3.5% (lower bacterial concentration) was obtained after lyophilization (SD temperature of 0°C for 6 h).

[00718] In general, no differences were observed between the frozen and the non-frozen material with the applied methods, indicating that long-term storage of the DS at –80°C may be possible, thereby eliminating the need for continuous manufacturing.

Example 6. Effect of Temperature and Time in Thawing Frozen Drug Substance Prior to Lyophilization.

[00719] Temperature and the time for thawing can impact stability. Identifying appropriate conditions for thawing frozen drug substance allows freezing and holding of the drug substance prior to lyophilization. Ensuring high-quality healthy cells coming out of thaw ensures that the resulting lyophilized drug product is also of sufficient quality.

6.1 Freeze-Thawing (FT) of the Bulk Drug Substance

[00720] Throughout development, various studies evaluated the ability to store Drug Substance (DS) at -80°C, thaw and compound manufacture a Lyophilized Drug Product (DP) batch at a later date. This involves a freeze-thaw of the DS. A freeze/thaw cycle was defined as the complete thawing of the DS followed by storage at -80°C for a minimum of 24 hours), until no ice crystals remain. The stress from the freeze-thaw could adversely impact the product

quality (e.g. VCC, % live) of the lyophilized product. Hence, a series of experiments were performed to determine the optimal storage conditions and thawing procedure for the DS.

6.2 Evaluation of DS Container (Bag vs. Bottle)

[00721] Early studies evaluated DS that was stored in bottles (Vibalogics Experiments Lyo8, Lyo12, and Lyo16, and Coriolis WP2A, WP2B, and WP3). Other studies thawed the DS at either 2-8°C (Vibalogics Lyo8 and Lyo12 and Coriolis WP2B and WP3) overnight or 37°C in a shorter span of time (Vibalogics Lyo16 and Coriolis WP2A, WP6, and WP7-Cycle 1). Later studies evaluated DS that was stored in a 1L LDPE bag and thawed at 37°C (WP7-Cycle 3).

6.3 Evaluation of DS Thaw Temperatures and DS Concentration

[00722] Since storage of the DS in LDPE bags is preferred over storage in bottles for GMP manufacturing, development efforts focused on conditions that resulted in higher VCC and % live profiles when stored in bags. A freeze/thaw cycle was defined as the complete thawing of the DS followed by storage at -80°C for a minimum of 24 hours. Freeze-thaw studies were completed to evaluate the Drug Substance VCC and % live over three FT cycles for a range of DS VCC levels (concentration) and thawing temperatures.

[00723] The FT studies evaluated DS at OD_{600} of 3.5 and 6.5. Approximately 1L of DS was filled into 1L LDPE bags and frozen at −80°C and subjected to three FT cycles at either 2-8°C, Room Temperature (RT) or 37°C. DS bags, at an OD_{600} of 6.5 or at an OD_{600} 3.5, were thawed in a 4°C refrigerator for 36 hours, at room temperature on a laboratory bench for approximately 12 hours or at 37°C in an incubator for ≤8 hours. After thawing completely, a sample was taken for analysis and the bags were placed in −80°C for a minimum of 24 hours prior to thawing at the respective conditions and re-freezing for the next cycle.

[00724] For the studied thawing conditions, VCC and viability values of BDS at an OD_{600} of 6.5 demonstrated a higher VCC and viability over the three freeze-thaw cycles relative to the BDS at an OD600 of 3.5 as shown in **FIG. 64** and **FIG. 65**.

[00725] The viability for the BDS at an OD_{600} of 3.5 decreased with multiple freeze thaw cycles for all thaw temperatures except the 37°C thaw. The 37°C thaw demonstrated freeze-thaw stability for both OD_{600} for 6.5 and 3.0.

[00726] The data demonstrate that thawing the DS at 37°C results in improved product quality over the range of VCC values evaluated. The data are further supported by the Coriolis experiments which evaluated DS stored as a concentrated pellet, in a bottle and thawed at 2-8°C which did not result in an acceptable accelerated stability profile. Based on the findings WP7 cycle 3 was performed to evaluate if the target storage and thaw conditions of the DS led to an improved in Lyo DP stability relative to DS that had not undergone a FT cycle. The results of WP7-cycle 3 which compared 2-8°C and −80°C stored DS with 1L fill in a 1L LDPE bag thawed at 37°C demonstrated comparable results demonstrating that it is feasible to freeze and thaw DS prior to lyophilization.

Example 7. Exemplary Lyophilization Conditions

7.1 Formulation

[00727] Table 29. Exemplary Formulation.

Target Buffer Composition for LYO Formulation				
Components	**Component Weights (g/L)**	**Weight of component (g/mol)**	**Molarity (mol/L)**	**Molarity (millimol/L)**
Potassium dihydrogen phosphate (KH_2PO_4)	0.20	136.08	0.001470	1.5
Disodium hydrogen phosphate (Na_2HPO_4)	1.14-1.15	141.96	0.008030	8.0
Sucrose	25.00	342.30	0.073036	73.0
Water for Injection (WFI)	Q.S to 1 L			

The Drug Product is reconstituted with normal saline.

7.2 Drug Substance Manufacturing and Thawing

[00728] The DS manufacturing consists of a single-use closed system of a 20 L culture bag for fermentation, a tangential flow filtration (TFF) manifold for concentration and buffer exchange and a container manifold for DS filling. DS can either be held at 2-8°C for up to three days or frozen at -80°C prior to compounding, filling, and lyophilization. DS target concentration 3.5-6.5 OD_{600}. Thawing of the DS is performed at 37°C in ≤ 8hours.

7.3 Lyophilization Cycle

[00729] Table 30. Exemplary Lyophilization Cycle.

Step	Phase		Item	Duration [hh:mm]	Temp, T_s of Shelves [°C]	Vacuum [mbar]
NA	Preparation		Warm up of Lyo, placement of temperature and Rx sensors	NA	4	Off
1	Load		Loading of Shelves	NA	4	Off
2	Ramp	Freezing	Ramp samples to -4°C (1.0°C/min)	00:49	-45	Off
3	Hold		Hold at -45°C	02:00	-45	Off
4	Ramp	Primary Drying	Vacuum*	00:01	-45	0.09
5	Hold		Vacuum*	00:15	-45	0.09
6	Ramp		Heating Ramp (1.0°C/min)	00:27	-18	0.09
7	Hold		Stable Shelf Temp	>tbc**	-18	0.09
9	Ramp	Secondary Drying	Heating Ramp (0.2°C/min)	01:30	0	0.09
9	Hold		Stable Shelf Temp	06:00	0	0.09
10	Hold		Hold at 0°C until unloading	NA	0	1.0***
10a			Ventilation and manual vial closure (N_2 sparging)	NA	0	500mbar

*Pirani vacuum sensor is the process controlling probe
**End of primary drying is defined here as 14 hours after the T_P P100 probe for the cold spots(s) has crossed the T_s set-point of 18°C.
***Only done to avoid vacuum pull after the cycle is finished during the night

7.4 Residual Moisture Target

[00730] The residual moisture target is >3.0% at release.

7.4 ADXS DS Platform Process Description

[00731] Fermentation is carried out within a single-use closed system provided by rocking wave motion bioreactor technology. The single-use closed system consists of a product culture bag for fermentation, a tangential flow filtration (TFF) system for concentration and buffer exchange, and a product manifold for drug substance container filling. Each of these components are sterilized by gamma irradiation and received in accordance with site quality systems.

[00732] The platform uses the rocking wave motion technology for fermentation. This technology offers the ability to control the entire processing operation in a closed system. The bulk DS is harvested by TFF using a single-use hollow fiber module and single-use disposable filtration path.

[00733] The composition of the fermentation media and the pH control solution (1 M sodium hydroxide) are provided in **Table 31**. The media for the inoculum is sterile-filtered through two

0.2 µm filters in series into a sterile 1 L glass bottle. The fermentation media is sterile-filtered through two 0.2 µm filters in series into a sterile 10 L glass bottle.

[00734] Table 31. Fermentation Media Formulation Table.

Formulation	Components	Component Weights
Fermentation Media (1 kg)	Tryptic Soy Broth	1 kg
	D (+) Glucose (monohydrate)	11.11 g
pH Control Solution (1M NaOH)	Sodium Hydroxide pellets	40 g
	WFI	1000 g

[00735] The culture bag is pre-connected with probes for dissolved oxygen (DO) and pH monitoring. It is then aseptically filled with 5 L of fermentation medium. The culture bag is inflated with 0.2 µm filtered compressed O_2 and air.

[00736] Filtered (0.2 µm) compressed air/O_2 is continuously fed during propagation at a rate of 1.0 L/minute, the O_2 flow set point is 50%, and is removed through a vent port. The rocking angle is set at 10°. The DO control is set to speed with a rocking rate between 18-36 rpm. The pH control bottle is aseptically connected to the culture bag. During propagation, the process is automatically monitored and controlled for temperature, pH and dissolved oxygen by an integrated controlling system.

[00737] A pre-culture is initiated from the working cell bank by pipetting 1 mL of the WCB into 170 mL of fermentation media and grown for approximately 10 hours until an OD_{600} of $\geq$ 3.5 is reached. The pre-culture is used to inoculate the production culture by aseptically transferring it to the culture bag. Growth is proceeded to an $OD_{600} \geq 7.5$. When the OD_{600} reaches the target value, the culture bag is connected, using Ready Mate connectors to the sterile TFF manifold for concentration and diafiltration against the formulation buffer. The TFF module uses a 0.2 µm pore size hollow fiber filter, meeting the low shear requirements of cell separation applications. A peristaltic pump is used to feed the fermentation culture into the TFF system. The bulk culture in the recirculation loop is initially set to a flow rate of approximately 75 rpm (approximately 4.5 L/min). The fermentation broth is concentrated 5-fold to a mass of approximately 1000 g. A permeate pump is used and set initially at 20% (approximately 275 mL/min).

[00738] The diafiltration/washing of the harvest concentrate is performed with ≥ 7 diavolumes. The retentate drug substance is sampled from the TFF assembly using an in-process sampling manifold. The OD_{600} of the sample is measured and used to calculate the dilution

volume needed to reach a target OD_{600} of ≤ 6.5. The required amount of formulation buffer is pumped into the retentate bag to dilute the retentate to the required concentration. All volume transfers are controlled by weight change in the respective bags in addition to the complete TFF assembly to control volume transfers. The retentate is sampled and measured to confirm the OD_{600} is ≤ 6.5. If the OD_{600} has not been sufficiently diluted, it may be further diluted. The DS is then distributed into approximately 1 L aliquots into product bags.

[00739] Each bag is heat-sealed for removal from the assembly. Each bag is individually labeled with the appropriate information and then stored at -70 ± 15°C.

7.6 Drug Substance Process Flow Diagram

[00740] *See* **FIG. 20**.

7.7 Drug Product Process Flow Diagram

[00741] *See* **FIG. 21**.

WE CLAIM:

1. A method for producing a lyophilized composition comprising a *Listeria* strain, comprising:

(a) providing a composition comprising a *Listeria* strain in a formulation comprising a buffer and sucrose,

wherein the *Listeria* strain is a *Listeria monocytogenes* strain, and

wherein the formulation comprises 1% to 5% w/v sucrose;

(b) cooling the composition provided in step (a) at a holding temperature between −32°C and −80°C in a freezing step;

(c) exposing the composition produced by step (b) to a vacuum at a holding temperature between −10°C and −30°C in a primary drying step; and

(d) exposing the composition produced by step (c) to a vacuum at a holding temperature between −5°C and 25°C in a secondary drying step,

whereby the lyophilized composition is produced, and wherein the residual moisture in the lyophilized composition is between 1% and 5%.

2. The method of claim 1, wherein prior to step (a), a stress response is induced in the *Listeria* strain by exposing the *Listeria* strain to a temperature of 0°C to 4°C.

3. The method of claim 1, wherein prior to step (a), a stress response is not induced in the *Listeria* strain by exposing the *Listeria* strain to a temperature of 0°C to 4°C.

4. The method of any preceding claim, wherein the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that is thawed prior to step (a).

5. The method of claim 4, wherein the concentration of the frozen *Listeria* strain being thawed is between 1x10E9 to 1x10E10 colony forming units (CFU) per milliliter.

6. The method of claim 4 or 5, wherein the frozen *Listeria* strain is thawed at:

2°C to 37°C;

20°C to 37°C;
32°C and 37°C; or
37°C.

7. The method of any one of claims 4-6, wherein the frozen *Listeria* strain is thawed for no more than 8 hours and/or held at 2°C to 8°C for no more than 24 hours after thawing.

8. The method of any one of claims 1-3, wherein the *Listeria* strain used in the composition in step (a) is freshly cultured prior to step (a).

9. The method of any preceding claim, wherein the buffer is a phosphate buffer.

10. The method of any preceding claim, wherein the formulation comprises:
2% to 3% w/v sucrose; or
2.5% w/v sucrose.

11. The method of any preceding claim, wherein the formulation comprises 1x10E9 to 1x10E10 colony forming units (CFU) of *Listeria* per milliliter.

12. The method of any preceding claim, wherein the formulation does not comprise one or more of trehalose, monosodium glutamate (MSG), and recombinant human serum albumin (rHSA).

13. The method of claim 12, wherein the formulation does not comprise trehalose, MSG, or rHSA.

14. The method of any preceding claim, wherein the holding temperature in the freezing step (b) is between −40°C and −50°C or −45°C.

15. The method of any preceding claim, wherein the freezing step (b) comprises decreasing the temperature to the holding temperature at a rate of 1°C per minute.

16. The method of any preceding claim, wherein the cooling in the freezing step (b) is for 2 hours to 4 hours or 2 hours.

17. The method of any preceding claim, wherein the holding temperature in the primary drying step (c) is:

between −12°C and −22°C;

between −17°C and −19°C; or

−18°C.

18. The method of any preceding claim, wherein the primary drying step (c) comprises increasing the temperature to the holding temperature at a rate of 1°C per minute.

19. The method of any preceding claim, wherein the primary drying step (c) is for 25 hours to 35 hours.

20. The method of any preceding claim, wherein the end of the primary drying step (c) is 12 to 16 hours after the composition has reached holding temperature.

21. The method of any preceding claim, wherein the primary drying step (c) is at a vacuum pressure of 0.09 mbar.

22. The method of any preceding claim, wherein the holding temperature in the secondary drying step (d) is:

between −5°C and 20°C;

between −5°C and 5°C; or

0°C.

23. The method of any preceding claim, wherein the secondary drying step (d):

comprises increasing the temperature to the holding temperature at a rate of 0.2°C per minute;

is for 1 hour to 10 hours;

comprises holding the composition at the holding temperature for 2 hours to 6 hours or 5 hours to 6 hours; and/or

is at a vacuum pressure of 0.09 mbar.

24. The method of any preceding claim, wherein the residual moisture in the lyophilized composition is:

between 2% and 4%;

at least 2.5%; or

at least 3%.

25. The method of any preceding claim, wherein the lyophilized composition shows:

at least 60% viability after storage at between −20°C and 4°C for 12 months;

at least 75% viability after storage at between −20°C and 4°C for 12 months; or

at least 80% viability after storage at between −20°C and 4°C for 12 months.

26. The method of any preceding claim, wherein the buffer is a phosphate buffer, and

wherein the formulation comprises 2% to 3% w/v sucrose, and

wherein the formulation does not comprise trehalose, MSG, or rHSA, and

wherein the formulation comprises 1x10E9 to 1x10E10 colony forming units (CFU) of *Listeria* per milliliter, and

wherein the holding temperature in the freezing step (a) is between −40°C and −50°C, and

wherein the holding temperature in the primary drying step (c) is between −17°C and −19°C, and

wherein the holding temperature in the secondary drying step (d) is between −1°C and 1°C, and

wherein the residual moisture in the lyophilized composition is between 2.5% and 4%.

27. The method of 26, wherein the *Listeria* strain used in the composition in step (a) is a frozen *Listeria* strain that is thawed prior to step (a), and

wherein the concentration of the frozen *Listeria* strain being thawed is between 1x10E9 to 1x10E10 colony forming units (CFU) per milliliter, and

wherein the frozen *Listeria* strain is thawed at 37°C, and

wherein the frozen *Listeria* strain is thawed for no more than 8 hours, and

wherein the frozen *Listeria* strain is held at 2°C to 8°C for no more than 24 hours after thawing.

28. The method of any preceding claim, wherein the *Listeria* strain is a recombinant *Listeria* strain comprising a nucleic acid comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a PEST-containing peptide fused to a disease-associated antigenic peptide.

29. The method of claim 28, wherein the recombinant *Listeria* strain is:

an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *prfA*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding a D133V PrfA mutant protein; or

an attenuated *Listeria monocytogenes* strain comprising a deletion of or inactivating mutation in *actA*, *dal*, and *dat*, wherein the nucleic acid is in an episomal plasmid and comprises a second open reading frame encoding an alanine racemase enzyme or a D-amino acid aminotransferase enzyme, and wherein the PEST-containing peptide is an N-terminal fragment of LLO.