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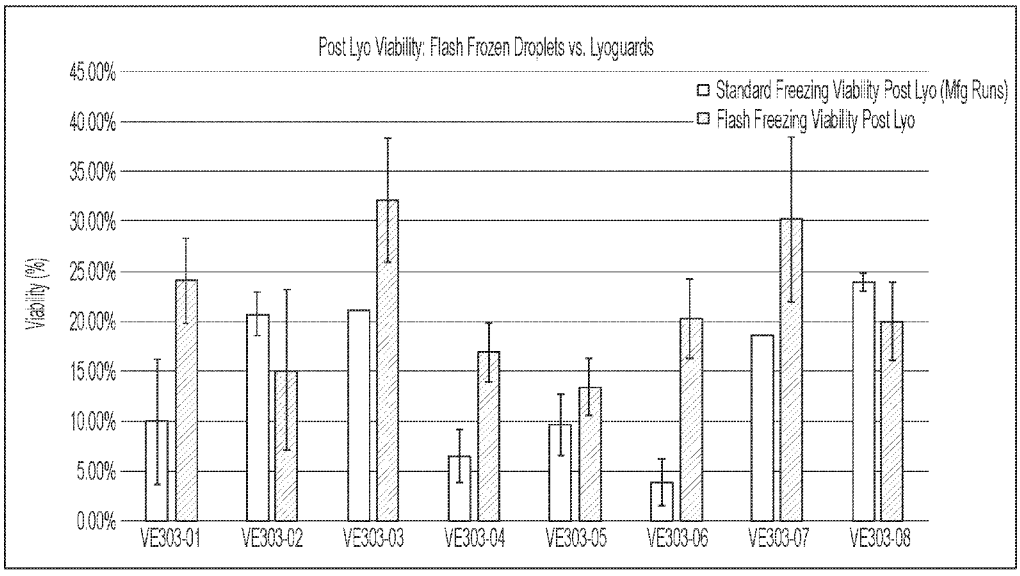

Figure 1

(57) Abstract: The disclosure provides methods and compositions for the preservation of bacteria. Aspects of the present disclosure provide methods of preparing a preserved bacterial composition comprising flash freezing a bacterial composition and lyophilizing the flash frozen bacterial composition to produce a preserved bacterial composition. In some embodiments, the bacterial composition comprises one or more bacterial strains. In some embodiments, the one or more bacterial strains comprise one or more anaerobic bacterial strains. In some embodiments, the anaerobic bacterial strains are strict anaerobic bacteria.

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METHODS AND COMPOSITIONS FOR PRESERVING BACTERIA

RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/901,205, filed September 16, 2019. The entire contents of the referenced application are incorporated by reference herein.

FIELD OF THE INVENTION

The disclosure provides methods and compositions for the preservation of bacteria.

BACKGROUND

The human intestinal microbiome includes a large number of microorganisms. A significant number of these microorganisms are anaerobic bacteria. Compositions that include anaerobic bacteria that originated from the human intestinal microbiome have shown potential in the treatment of human disease (See *e.g.,* Atarashi et al., *Nature* 500, 232, 2013; Atarashi et al., *Cell* 163, 1, 2015; Mathewson et al., *Nature Immunology* 17, 505, 2016). Anaerobic bacteria are challenging to preserve because of their sensitivity to oxygen. Improved compositions and methods for the preservation of anaerobic bacteria are needed therefore.

SUMMARY

Aspects of the present disclosure provide methods of preparing a preserved bacterial composition comprising flash freezing a bacterial composition and lyophilizing the flash frozen bacterial composition to produce a preserved bacterial composition. In some embodiments, the bacterial composition comprises one or more bacterial strains. In some embodiments, the one or more bacterial strains comprise one or more anaerobic bacterial strains. In some embodiments, the anaerobic bacterial strains are strict anaerobic bacteria.

In some embodiments, the bacterial composition comprises one or more bacterial strains belonging to the class Clostridia. In some embodiments, the one or more bacterial strains belong to the family Clostridiaceae. In some embodiments, the bacteria comprise one or more bacterial strains belonging to the genus *Clostridium*. In some embodiments, the bacterial composition comprises one or more bacterial strains selected from the group consisting of *Clostridium bolteae, Anaerotruncus colihominis, Eubacteriaum fissicatena, Clostridium symbiosum, Blautia producta, Dorea longicatena, Clostridium innocuum,* and

Flavinofractor plautti. In some embodiments, the bacterial composition comprises one or more bacterial strains comprising 16S rDNA sequences having at least 97% sequence identity with the nucleic acid sequences selected from the group consisting of SEQ ID NOs: 1-8.

In some embodiments, the method further comprises culturing the bacterial composition. In some embodiments, the bacterial composition is washed and resuspended in a formulation buffer. In some embodiments, the flash freezing is performed by contacting the bacterial composition with a super-cooled surface. In some embodiments, the flash freezing is performed by contacting the bacterial composition with liquid nitrogen.

In some embodiments, the bacterial composition has a symmetrical shape. In some embodiments, the symmetrical shape is a symmetrical frozen droplet. In some embodiments, the preserved bacterial composition is subjected to a temperature of -80°C.

In some embodiments, the lyophilizing comprises a primary drying step and a secondary drying step. In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of -10°C and under a pressure of 70 mTorr. In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a temperature of +20°C and under a pressure of 70 mTorr.

In some embodiments, the method further comprises determining a level of viability in the preserved bacterial composition after lyophilizing. In some embodiments, the level of viability in the preserved bacterial composition is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, or at least 40% of colony forming units of the bacteria over a period of time. In some embodiments, the period of time is at least 1 week, at least 2 weeks, at least 4 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year or more.

These and other aspects of the invention, as well as various embodiments thereof, will become more apparent in reference to the drawings and detailed description of the invention.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWING

The accompanying drawing is not intended to be drawn to scale. The figure is illustrative only and is not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in the drawing. In the drawing:

Figure 1 shows the post-lyophilization viability of the indicated bacterial strains. The percent post-lyophilization viability (% viability on the y-axis) is calculated based on the enumerated colony forming units following lyophilization relative to the enumerated colony forming units following harvest of the bacterial culture. For each of the indicated bacterial strains, the left column corresponds to viability following the standard freezing process (*i.e.*, freeze-drying tray) prior to lyophilization, and the right column corresponds to viability following the flash freezing droplets and lyophilizing methods described herein.

DETAILED DESCRIPTION

The preservation of bacterial compositions, including anaerobic bacteria, has been challenging. While bacteria can be frozen down and regrown on plates or in solution, it has been difficult to standardize this process. There is a need to preserve bacteria that can be used for therapeutic purposes. Preservation processes, such as cryopreservation and lyophilization, have been well established for aerobic bacteria, and many factors that affect survival and recovery of aerobic bacteria in the preservation process are understood (Prakash et al. *FEMS Microbiol Lett* (2013)339:1-9). For bacterial products that rely on viable bacteria, enhancing the level of viability of bacteria following preservation processes have the potential to reduce costs associated with production of such products. Given that bacteria, such as anaerobic bacterial strains obtained from the human intestinal microbiome have shown potential in the treatment of human disease, improved methods for preserving bacteria that allow for high levels of bacterial recovery are needed.

Described herein are methods of preserving bacterial compositions comprising flash freezing a bacterial composition and lyophilizing the flash frozen bacterial composition to produce a preserved bacterial composition. The ability to freeze and store the flash frozen bacterial compositions allows greater flexibility with the preservation process. In some embodiments, the methods described herein are used for the preservation of anaerobic bacteria.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the

drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Provided herein are compositions and methods for the preservation of bacteria. In one aspect, the methods provided herein allow for preservation of bacterial compositions. In some embodiments, the methods described herein are used for the preservation of anaerobic bacteria. The compositions allow the bacteria to go through a freeze-dry cycle with minimal loss to viability. In some embodiments, the bacterial composition includes bacteria. In some embodiments, the bacterial composition includes one or more bacterial strains. In some embodiments, the bacterial composition includes a single bacterial strain. In some embodiments, the bacterial composition comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 or more bacterial strains (*e.g.*, purified bacterial strains).

In some embodiments, the bacterial composition comprises one or more anaerobic bacterial strains (*e.g.*, strict anaerobic bacteria). In some embodiments of the compositions provided herein, the anaerobic bacteria are strict anaerobic bacteria.

In some embodiments, the bacterial composition comprises one or bacterial strains belonging to the class Clostridia. In some embodiments, one or more bacterial strains are from the family Clostridiaceae. In some embodiments, the bacteria are from the genus *Clostridium*. In some embodiments, the bacteria belong to *Clostridium* cluster IV, XIVa, XVI, XVII, or XVIII. In some embodiments, the bacteria belong to *Clostridium* cluster IV, XIVa, or XVII. In some embodiments, the bacteria belong to *Clostridium* cluster IV or XIVa.

In some embodiments, the bacterial composition includes one or more of the following bacterial strains: *Clostridium bolteae*, *Anaerotruncus colihominis*, *Sellimonas intestinalis*, *Clostridium symbiosum*, *Blautia producta*, *Dorea longicatena, Erysipelotrichaceae bacterium* and *Flavinofractor plautii*. Bacterial strains *Clostridium bolteae*, *Anaerotruncus colihominis*, *Sellimonas intestinalis*, *Clostridium symbiosum*, *Blautia producta*, *Dorea longicatena, Erysipelotrichaceae bacterium* and *Flavinofractor plautii* are described, for instance, in PCT Publication No. WO 2017/218680, which is incorporated by reference in its entirety. The strains are also depicted in Table 1. It should be appreciated that alternative strain names, *e.g.*, as depicted in Table 1, may be used as well.

In some embodiments, the bacterial composition includes one or more of the following bacterial strains: *Clostridium bolteae 90A9*, *Anaerotruncus colihominis DSM17241*, *Sellimonas intestinalis, Clostridium bolteae*, *Anaerotruncus colihominis*, *Sellimonas intestinalis*, *Clostridium symbiosum*, *Blautia producta*, *Dorea longicatena, Erysipelotrichaceae bacterium* and *Flavinofractor plautii*. In some embodiments, the bacterial composition includes two or more (*e.g.*, 2, 3, 4, 5 6, 7, or 8) of the following bacterial strains: *Clostridium bolteae*, *Anaerotruncus colihominis*, *Sellimonas intestinalis*, *Clostridium symbiosum*, *Blautia producta*, *Dorea longicatena, Erysipelotrichaceae bacterium* and *Flavinofractor plautii*. In some embodiments, the bacterial composition includes *Clostridium bolteae.* In some embodiments, the bacterial composition includes *Anaerotruncus colihominis.* In some embodiments, the bacterial composition includes *Sellimonas intestinalis.* In some embodiments, the bacterial composition includes *Clostridium symbiosum.* In some embodiments, the bacterial composition includes *Blautia producta.* In some embodiments, the bacterial composition includes *Dorea longicatena.* In some embodiments, the bacterial composition includes *Erysipelotrichaceae bacterium.* In some embodiments, the bacterial composition includes *Flavinofractor plautii*.

In one aspect, as shown herein (*e.g.*, in the Example) the methods provided herein allow for the preservation of anaerobic bacterial strains. Anaerobic strains that can be used in the methods of the current invention include bacterial strains that are used in therapeutic consortia, such as described for instance in PCT Publication Nos. WO2013/080561, WO2015/156419, WO2018/117263, WO2017/218680, WO2019/094837, and WO2019/118515. In one aspect, as shown herein (*e.g.,* in the Example) the methods provided herein allow for the preservation of anaerobic bacterial strains belonging to *Clostridium* cluster IV, XIVa, or XVII. In one aspect, as shown herein (*e.g.*, in the Example) the methods provided herein allow for the preservation of anaerobic bacterial strains *Clostridium bolteae*, *Anaerotruncus colihominis*, *Sellimonas intestinalis*, *Clostridium symbiosum*, *Blautia producta*, *Dorea longicatena, Erysipelotrichaceae bacterium* and *Flavinofractor plautii*. The exemplary bacterial strains can also be identified by their 16s rRNA sequences (SEQ ID NOs: 1-8). Identifying bacteria by their sequences furthermore allows for the identification of additional bacterial strains that are identical or highly similar to the exemplified bacteria. For instance, the 16s rRNA sequences of bacterial strains were used to identify the closest relative (based on percent identity) through whole genome sequencing and by comparing these sequences with 16S databases (Table 1). In addition, based on whole genome sequencing and comparing of the whole genome to whole genome

databases, the bacterial strains having 16S rRNA sequences provided by SEQ ID NOs: 1-8 are most closely related to the following bacterial species: *Clostridium bolteae* 90A9, *Anaerotruncus colihominis* DSM 17241, *Dracourtella massiliensis* GD1, *Clostridium symbiosum* WAL-14163, *Clostridium bacterium* UC5.1-1D4, *Dorea longicatena* CAG:42, *Erysipelotrichaceae bacterium* 21_3, and *Clostridium orbiscindens* 1_3_50AFAA (*see, e.g.*, Table 1). Thus, in one aspect it should be appreciated that each row of Table 1, the bacterial strains are highly similar and/or are identical. In some embodiments, in context of the instant disclosure the names of bacterial strains within a row of Table 1 can be used interchangeably.

Table 1: Examples of Bacterial species of the compositions disclosed herein

Strain number	SEQ ID NO:	Closest species based on Sanger sequencing of 16S region	Closest species based on Consensus SEQ ID # of 16S region as compared with 16S database	Closest species based on WGS compared versus WG databases	Additional closely related sequences	*Clostridium* cluster
VE303-1	1	*Clostridium bolteae*	*Clostridium bolteae*	*Clostridium bolteae* 90A9		XIVa
VE303-2	2	*Anaerotruncus colihominis*	*Anaerotruncus colihominis*	*Anaerotruncus colihominis* DSM 17241		IV
VE303-3	3	*Eubacterium fissicatena*	*Dracourtella massiliensis*	*Dracourtella massiliensis* GD1	*Ruminococcus torques*; *Sellimonas intestinalis*	XIVa
VE303-4	4	*Clostridium symbiosum*	*Clostridium symbiosum*	*Clostridium symbiosum* WAL-14163		XIVa
VE303-5	5	*Blautia producta*	*Blautia producta*	*Clostridium bacterium* UC5.1-1D4	*Blautia producta* ATCC 27340	XIVa
VE303-6	6	*Dorea longicatena*	*Dorea longicatena*	*Dorea longicatena* CAG:42		XIVa
VE303-7	7	*Clostridium innocuum*	*Clostridium innocuum*	*Erysipelotrichaceae bacterium* 21_3		XVII
VE303-8	8	*Flavinofractor plautii*	*Flavinofractor plautii*	*Clostridium orbiscindens* 1_3_50AFAA	*Subdolinogranulum*	IV

Aspects of the disclosure relate to bacterial strains with 16S rDNA sequences that have sequence identity to a nucleic acid sequence of any one of the sequences of the bacterial strains or species described herein. The terms "identical," percent "identity" in the context of two or more nucleic acids or amino acid sequences, refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity) over a specified region of a nucleic acid or amino acid sequence or over an entire sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length. In some embodiments, the identity exists over the length the 16S rRNA or 16S rDNA sequence.

In some embodiments, the bacterial composition includes one or more bacterial strain that has at least 60%, at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% or up to 100% sequence identity to any one of the strains or bacterial species described herein over a specified region or over the entire sequence. It would be appreciated by one of skill in the art that the term "sequence identity" or "percent sequence identity" in the context of two or more nucleic acid sequences or amino acid sequences, refers to a measure of similarity between two or more sequences or portion(s) thereof.

In some embodiments, the bacterial composition includes one or more bacterial strains, wherein the one or more bacterial strains include one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 80%, at least 81%,

at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9%, or up to 100% sequence identity with nucleic acid sequences SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8.

In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO: 1. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO: 2. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO: 3. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO:4. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO:5. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO:6. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO:7. In some embodiments, the bacterial composition includes one bacterial strain, wherein the bacterial strain includes one or more 16s rDNA sequences having at least 97% sequence identity with nucleic acid sequences SEQ ID NO:8.

Additionally, or alternatively, two or more sequences may be assessed for the alignment between the sequences. The terms " alignment " or percent " alignment " in the context of two or more nucleic acids or amino acid sequences, refer to two or more sequences or subsequences that are the same. Two sequences are “substantially aligned” if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical) over a specified region of the nucleic acid or amino acid sequence or over the entire sequence, when compared and aligned for maximum correspondence over a

comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the alignment exists over a region that is at least about 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length. In some embodiments, the identity exists over the length the 16S rRNA or 16S rDNA sequence.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Methods of alignment of sequences for comparison are well known in the art. See, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* (1970) 48:443, by the search for similarity method of Pearson and Lipman. *Proc. Natl. Acad. Sci. USA* (1998) 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group. Madison, WI), or by manual alignment and visual inspection (see. e.g., Brent et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (Ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *Nuc. Acids Res.* (1977) 25:3389-3402, and Altschul et al., *J. Mol. Biol.* (1990) 215:403-410, respectively.

It should be appreciated that the terms "bacteria" and "bacterial strains" as used herein are interchangeable.

As used herein, the term "isolated" refers to a bacteria or bacterial strain that has been separated from one or more undesired component, such as another bacterium or bacterial strain, one or more component of a growth medium, and/or one or more component of a sample, such as a fecal sample. In some embodiments, the bacteria are substantially isolated from a source such that other components of the source are not detected.

In some embodiments, the bacterial strains are grown up from a single colony. In some embodiments, the bacterial strains are purified bacterial strains. As used herein, the term "purified" refers to a bacterial strain or composition comprising such that has been separated from one or more components, such as contaminants. In some embodiments, the bacterial strain is substantially free of contaminants. In some embodiments, one or more bacterial strains of a composition may be independently purified from one or more other bacteria produced and/or present in a culture or a sample containing the bacterial strain. In some embodiments, a bacterial strain is isolated or purified from a sample and then cultured under the appropriate conditions for bacterial replication, *e.g.,* under anaerobic culture

conditions. The bacteria that is grown under appropriate conditions for bacterial replication can subsequently be isolated/purified from the culture in which it is grown.

The bacterial strains of the composition can be manufactured using fermentation techniques well known in the art. In some embodiments, the active ingredients are manufactured using anaerobic fermenters, which can support the rapid growth of anaerobic bacterial strains. The anaerobic fermenters may be, for example, stirred tank reactors or disposable wave bioreactors. Culture media such as BL media and EG media, or similar versions of these media devoid of animal components, can be used to support the growth of the bacterial species. In some embodiments, the bacterial composition is grown to a desired growth phase prior to flash freezing. In some embodiments, the bacterial composition is grown to a desired cell density prior to flash freezing. In some embodiments, the cell density is a desired optical density (e.g., OD_{600}) of the bacterial strain.

The bacterial product can be purified and concentrated from the fermentation broth by traditional techniques, such as centrifugation and filtration. Generally, the bacteria are pelleted prior to subjecting the bacterial composition to flash freezing. In some embodiments, the bacterial composition is washed prior to flash freezing. As used herein, the term "wash" or "washing" refers to series of steps to isolate bacterial cells and remove residual undesired components (*e.g.*, cell debris, components of growth media). In some embodiments, the method involves isolating bacterial cells from a culture (*e.g.*, growth media), resuspending the isolated bacterial cells in a wash buffer, and isolating the bacterial cells from the wash buffer by traditional techniques, such as centrifugation and filtration. In some embodiments, the isolated bacterial cells are resuspended in formulation buffer. In some embodiments, the method involves washing the bacterial composition and resuspending the bacterial composition in a formulation buffer.

In some embodiments, the formulation buffer comprises a lyoprotectant, a nutrient, a buffer, and an antioxidant. Example formulation buffers are described in PCT Publication No. WO 2018/081550, which is incorporated by reference herein in its entirety. In some embodiments, the disclosure provides a composition comprising a lyoprotectant, a nutrient, an antioxidant, and a buffer. In some embodiments, the formulation buffer comprises sucrose, yeast extract, L-cysteine, histidine, and magnesium chloride. In some embodiments, the formulation buffer comprises7% sucrose, 0.1% yeast extract, 0.05% L-cysteine, 20 mM histidine, and 0.01% magnesium chloride. In some embodiments, the formulation buffer contains sodium metabisulfite. In some embodiments, the formulation buffer contains 0.05% sodium metabisulfite.

In some embodiments, the bacterial compositions disclosed herein contain about 10, about 10^2, about 10^3, about 10^4, about 10^5, about 10^6, about 10^7, about 10^8, about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, about 10^{13} or more bacteria. In some embodiments, the bacterial compositions disclosed herein contain about 10, about 10^2, about 10^3, about 10^4, about 10^5, about 10^6, about 10^7, about 10^8, about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, about 10^{13} or more bacteria per milliliter. It should be appreciated that some of the bacteria may not be viable. In some embodiments, the bacterial compositions disclosed herein contain about 10, about 10^2, about 10^3, about 10^4, about 10^5, about 10^6, about 10^7, about 10^8, about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, about 10^{13} or more colony forming units (cfus) of bacteria. In some embodiments, the bacterial compositions disclosed herein contain about 10, about 10^2, about 10^3, about 10^4, about 10^5, about 10^6, about 10^7, about 10^8, about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, about 10^{13} or more colony forming units (cfus) of bacteria per milliliter.

In some embodiments, the bacterial compositions disclosed herein contain between 10 and 10^{13}, between 10^2 and 10^{13}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{13}, between 10^8 and 10^{13}, between 10^9 and 10^{13}, between 10^{10} and 10^{13}, between 10^{11} and 10^{13}, between 10^{12} and 10^{13}, between 10 and 10^{12}, between 10^2 and 10^{12}, between 10^3 and 10^{12}, between 10^4 and 10^{12}, between 10^5 and 10^{12}, between 10^6 and 10^{12}, between 10^7 and 10^{12}, between 10^8 and 10^{12}, between 10^9 and 10^{12}, between 10^{10} and 10^{12}, between 10^{11} and 10^{12}, between 10 and 10^{11}, between 10^2 and 10^{11}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{11}, between 10^8 and 10^{11}, between 10^9 and 10^{11}, between 10^{10} and 10^{11}, between 10 and 10^{10}, between 10^2 and 10^{10}, between 10^3 and 10^{10}, between 10^4 and 10^{10}, between 10^5 and 10^{10}, between 10^6 and 10^{10}, between 10^7 and 10^{10}, between 10^8 and 10^{10}, between 10^9 and 10^{10}, between 10 and 10^9, between 10^2 and 10^9, between 10^3 and 10^9, between 10^4 and 10^9, between 10^5 and 10^9, between 10^6 and 10^9, between 10^7 and 10^9, between 10^8 and 10^9, between 10 and 10^8, between 10^2 and 10^8, between 10^3 and 10^8, between 10^4 and 10^8, between 10^5 and 10^8, between 10^6 and 10^8, between 10^7 and 10^8, between 10 and 10^7, between 10^2 and 10^7, between 10^3 and 10^7, between 10^4 and 10^7, between 10^5 and 10^7, between 10^6 and 10^7, between 10 and 10^6, between 10^2 and 10^6, between 10^3 and 10^6, between 10^4 and 10^6, between 10^5 and 10^6, between 10 and 10^5, between 10^2 and 10^5, between 10^3 and 10^5, between 10^4 and 10^5, between 10 and 10^4, between 10^2 and 10^4, between 10^3 and 10^4, between 10 and 10^3, between 10^2 and 10^3, or between 10 and 10^2 total bacteria. In some embodiments, the compositions disclosed herein contain between 10 and 10^{13}, between 10^2 and 10^{13}, between 10^3 and 10^{13}, between 10^4 and

10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{13}, between 10^8 and 10^{13}, between 10^9 and 10^{13}, between 10^{10} and 10^{13}, between 10^{11} and 10^{13}, between 10^{12} and 10^{13}, between 10 and 10^{12}, between 10^2 and 10^{12}, between 10^3 and 10^{12}, between 10^4 and 10^{12}, between 10^5 and 10^{12}, between 10^6 and 10^{12}, between 10^7 and 10^{12}, between 10^8 and 10^{12}, between 10^9 and 10^{12}, between 10^{10} and 10^{12}, between 10^{11} and 10^{12}, between 10 and 10^{11}, between 10^2 and 10^{11}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{11}, between 10^8 and 10^{11}, between 10^9 and 10^{11}, between 10^{10} and 10^{11}, between 10 and 10^{10}, between 10^2 and 10^{10}, between 10^3 and 10^{10}, between 10^4 and 10^{10}, between 10^5 and 10^{10}, between 10^6 and 10^{10}, between 10^7 and 10^{10}, between 10^8 and 10^{10}, between 10^9 and 10^{10}, between 10 and 10^9, between 10^2 and 10^9, between 10^3 and 10^9, between 10^4 and 10^9, between 10^5 and 10^9, between 10^6 and 10^9, between 10^7 and 10^9, between 10^8 and 10^9, between 10 and 10^8, between 10^2 and 10^8, between 10^3 and 10^8, between 10^4 and 10^8, between 10^5 and 10^8, between 10^6 and 10^8, between 10^7 and 10^8, between 10 and 10^7, between 10^2 and 10^7, between 10^3 and 10^7, between 10^4 and 10^7, between 10^5 and 10^7, between 10^6 and 10^7, between 10 and 10^6, between 10^2 and 10^6, between 10^3 and 10^6, between 10^4 and 10^6, between 10^5 and 10^6, between 10 and 10^5, between 10^2 and 10^5, between 10^3 and 10^5, between 10^4 and 10^5, between 10 and 10^4, between 10^2 and 10^4, between 10^3 and 10^4, between 10 and 10^3, between 10^2 and 10^3, or between 10 and 10^2 total bacteria per milliliter.

In some embodiments, the bacterial compositions disclosed herein contain between 10 and 10^{13}, between 10^2 and 10^{13}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{13}, between 10^8 and 10^{13}, between 10^9 and 10^{13}, between 10^{10} and 10^{13}, between 10^{11} and 10^{13}, between 10^{12} and 10^{13}, between 10 and 10^{12}, between 10^2 and 10^{12}, between 10^3 and 10^{12}, between 10^4 and 10^{12}, between 10^5 and 10^{12}, between 10^6 and 10^{12}, between 10^7 and 10^{12}, between 10^8 and 10^{12}, between 10^9 and 10^{12}, between 10^{10} and 10^{12}, between 10^{11} and 10^{12}, between 10 and 10^{11}, between 10^2 and 10^{11}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{11}, between 10^8 and 10^{11}, between 10^9 and 10^{11}, between 10^{10} and 10^{11}, between 10 and 10^{10}, between 10^2 and 10^{10}, between 10^3 and 10^{10}, between 10^4 and 10^{10}, between 10^5 and 10^{10}, between 10^6 and 10^{10}, between 10^7 and 10^{10}, between 10^8 and 10^{10}, between 10^9 and 10^{10}, between 10 and 10^9, between 10^2 and 10^9, between 10^3 and 10^9, between 10^4 and 10^9, between 10^5 and 10^9, between 10^6 and 10^9, between 10^7 and 10^9, between 10^8 and 10^9, between 10 and 10^8, between 10^2 and 10^8, between 10^3 and 10^8, between 10^4 and 10^8, between 10^5 and 10^8, between 10^6 and 10^8, between 10^7 and 10^8,

between 10 and 10^7, between 10^2 and 10^7, between 10^3 and 10^7, between 10^4 and 10^7, between 10^5 and 10^7, between 10^6 and 10^7, between 10 and 10^6, between 10^2 and 10^6, between 10^3 and 10^6, between 10^4 and 10^6, between 10^5 and 10^6, between 10 and 10^5, between 10^2 and 10^5, between 10^3 and 10^5, between 10^4 and 10^5, between 10 and 10^4, between 10^2 and 10^4, between 10^3 and 10^4, between 10 and 10^3, between 10^2 and 10^3, or between 10 and 10^2 colony forming units of bacteria. In some embodiments, the compositions disclosed herein contain between 10 and 10^{13}, between 10^2 and 10^{13}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{13}, between 10^8 and 10^{13}, between 10^9 and 10^{13}, between 10^{10} and 10^{13}, between 10^{11} and 10^{13}, between 10^{12} and 10^{13}, between 10 and 10^{12}, between 10^2 and 10^{12}, between 10^3 and 10^{12}, between 10^4 and 10^{12}, between 10^5 and 10^{12}, between 10^6 and 10^{12}, between 10^7 and 10^{12}, between 10^8 and 10^{12}, between 10^9 and 10^{12}, between 10^{10} and 10^{12}, between 10^{11} and 10^{12}, between 10 and 10^{11}, between 10^2 and 10^{11}, between 10^3 and 10^{13}, between 10^4 and 10^{13}, between 10^5 and 10^{13}, between 10^6 and 10^{13}, between 10^7 and 10^{11}, between 10^8 and 10^{11}, between 10^9 and 10^{11}, between 10^{10} and 10^{11}, between 10 and 10^{10}, between 10^2 and 10^{10}, between 10^3 and 10^{10}, between 10^4 and 10^{10}, between 10^5 and 10^{10}, between 10^6 and 10^{10}, between 10^7 and 10^{10}, between 10^8 and 10^{10}, between 10^9 and 10^{10}, between 10 and 10^9, between 10^2 and 10^9, between 10^3 and 10^9, between 10^4 and 10^9, between 10^5 and 10^9, between 10^6 and 10^9, between 10^7 and 10^9, between 10^8 and 10^9, between 10 and 10^8, between 10^2 and 10^8, between 10^3 and 10^8, between 10^4 and 10^8, between 10^5 and 10^8, between 10^6 and 10^8, between 10^7 and 10^8, between 10 and 10^7, between 10^2 and 10^7, between 10^3 and 10^7, between 10^4 and 10^7, between 10^5 and 10^7, between 10^6 and 10^7, between 10 and 10^6, between 10^2 and 10^6, between 10^3 and 10^6, between 10^4 and 10^6, between 10^5 and 10^6, between 10 and 10^5, between 10^2 and 10^5, between 10^3 and 10^5, between 10^4 and 10^5, between 10 and 10^4, between 10^2 and 10^4, between 10^3 and 10^4, between 10 and 10^3, between 10^2 and 10^3, or between 10 and 10^2 colony forming units of bacteria per milliliter.

In some embodiments of the bacterial compositions provided herein, the composition includes at least 1 x 10^8 colony forming units of bacteria per milliliter.

The methods described herein involve flash freezing a bacterial composition. As used herein, the term "flash freezing," also known to as "snap freezing," refers to a process by which the temperature of a bacterial composition is rapidly lowered to temperatures below -70°C, for example using liquid nitrogen or dry ice. In some embodiments, the flash freezing involves contacting the bacterial composition with a super-cooled surface. In some

embodiments, the flash freezing involves contacting the bacterial composition with a receptacle containing liquid nitrogen or dry ice. In some embodiments, the flash freezing involves contacting the bacterial composition with liquid nitrogen. In some embodiments, the bacterial composition is applied to liquid nitrogen forming droplets. In some embodiments, the droplets of the bacterial composition are collected from the liquid nitrogen. In some embodiments, the droplets of the bacterial composition are collected from the liquid nitrogen and transferred to a receptacle for lyophilization (*e.g.*, lyophilization vial).

Compositions that include bacterial strains can be lyophilized to preserve the bacterial strain. In some embodiments, the composition or the bacterial strains of the composition are lyophilized. Methods of lyophilizing compositions, including compositions comprising bacteria, are known in the art. See, *e.g.*, U.S. Patent 3,261,761; U.S. Patent 4,205,132; PCT Publication Nos. WO 2014/029578, WO 2012/098358, WO2012/076665, and WO2012/088261, herein incorporated by reference in their entirety. However, finding conditions that allow for the lyophilization of certain bacteria, such as anaerobic bacteria has been challenging. *See e.g.,* Peiren et al., *Appl Microbol Biotechnol* (2015) 99: 3559. It should be appreciated that in one aspect the methods of stabilization and preservation provided herein allow for the ability to generate compositions that allow for the manufacture of bacterial strains, in particular anaerobic bacterial strains. The methods described herein result in increased viability of lyophilized bacterial compositions.

Aspects of the disclosure provide methods of preparing a preserved bacterial composition involving flash freezing a bacterial composition and lyophilizing the flash frozen bacterial composition to produce a preserved bacterial composition. In general, lyophilization is a desiccation process to preserve a material, such as bacteria, involving freeze-drying. Water is removed from material by freezing the material and then placing the material under a vacuum, during which the ice undergoes sublimation. In some embodiments, the lyophilization cycle involves the steps of freezing, primary drying, and secondary drying. The term "temperature ramp rate" refers to the rate by which the temperature is adjusted between steps of the lyophilization cycle.

In some embodiments, the lyophilization cycle comprises a primary drying step and a secondary drying step, each of which involves subjecting the bacterial composition to a desired temperature and pressure.

In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about -30°C to +10°C, -20°C to 0°C, -15°C to -5°C, or -12°C to -7°C. In some embodiments, the primary drying step comprises subjecting

the flash frozen bacterial composition to a temperature of about -30°C, -29°C, -28°C, -27°C, -26°C, -25°C, -24°C, -23°C, -22°C, -21°C, -20°C, -19°C, -18°C, -17°C, -16°C, -15°C, -14°C, -13°C, -12°C, -11°C, -10°C, -9°C, -8°C, -7°C, -6°C, -5°C, -4°C, -3°C, -2°C, -1°C, 0°C, +1°C, +2°C, +3°C, +4°C, +5°C, +6°C, +7°C, +8°C, +9°C, or +10°C. In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about -10°C.

In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 50 mTorr to 90 mTorr, 60 mTorr to 80 mTorr, 65 mTorr to 75 mTorr, 60 mTorr to 70 mTorr, 55 mTorr to 75 mTorr, or 70 mTorr to 85 mTorr. In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 50 mTorr, 51 mTorr, 52 mTorr, 53 mTorr, 54 mTorr, 55 mTorr, 56 mTorr, 57 mTorr, 58 mTorr, 59 mTorr, 60 mTorr, 61 mTorr, 62 mTorr, 63 mTorr, 64 mTorr, 65 mTorr, 66 mTorr, 67 mTorr, 68 mTorr, 69 mTorr, 70 mTorr, 71 mTorr, 72 mTorr, 73 mTorr, 74 mTorr, 75 mTorr, 76 mTorr, 77 mTorr, 78 mTorr, 79 mTorr, 80 mTorr, 81 mTorr, 82 mTorr, 83 mTorr, 84 mTorr, 85 mTorr, 86 mTorr, 87 mTorr, 88 mTorr, 89 mTorr, or 90 mTorr. In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 70 mTorr.

In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about -10°C and a pressure of about 70 mTorr.

In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about 0°C to +40°C, +10°C to +30°C, +15°C to +25°C, or +17°C to +22°C. In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about 0°C, +1°C, +2°C, +3°C, +4°C, +5°C, +6°C, +7°C, +8°C , +9°C , +10°C, +11°C, +12°C, +13°C, +14°C, +15°C, +16°C, +17°C, +18°C, +19°C, +20°C, +21°C, +22°C, +23°C, +24°C, +25°C, +26°C, +27°C, +28°C, +29°C, +30°C, +31°C, +32°C, +33°C, +34°C, +35°C, +36°C, +37°C, +38°C, +39°C, or +40°C. In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about +20°C.

In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 50 mTorr to 90 mTorr, 60 mTorr to 80 mTorr, 65 mTorr to 75 mTorr, 60 mTorr to 70 mTorr, 55 mTorr to 75 mTorr, or 70 mTorr to 85 mTorr. In some embodiments, the secondary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 50 mTorr, 51 mTorr, 52 mTorr, 53 mTorr, 54 mTorr, 55 mTorr, 56 mTorr, 57 mTorr, 58 mTorr, 59 mTorr, 60 mTorr, 61 mTorr, 62

mTorr, 63 mTorr, 64 mTorr, 65 mTorr, 66 mTorr, 67 mTorr, 68 mTorr, 69 mTorr, 70 mTorr, 71 mTorr, 72 mTorr, 73 mTorr, 74 mTorr, 75 mTorr, 76 mTorr, 77 mTorr, 78 mTorr, 79 mTorr, 80 mTorr, 81 mTorr, 82 mTorr, 83 mTorr, 84 mTorr, 85 mTorr, 86 mTorr, 87 mTorr, 88 mTorr, 89 mTorr, or 90 mTorr. In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a pressure of about 70 mTorr.

In some embodiments, the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of about +20°C and a pressure of about 70 mTorr.

In some embodiments, the lyophilization cycle includes one or more steps having a temperature ramp rate between 0.5°C/min to 3°C/min. In some embodiments, the temperature ramp rate is 0.5°C/min, 0.6°C/min ,0.7°C/min, 0.8°C/min, 0.9°C/min, 1.0°C/min, 1.1°C/min, 1.2°C/min, 1.3°C/min, 1.4°C/min, 1.5°C/min, 1.6°C/min, 1.7°C/min, 1.8°C/min, 1.9°C/min, 2.0°C/min, 2.1°C/min, 2.2°C/min, 2.3°C/min, 2.4°C/min, 2.5°C/min, 2.6°C/min, 2.7°C/min, 2.8°C/min, 2.9°C/min, or 3.0°C/min. In some embodiments, the lyophilization cycle includes one or more steps having a temperature ramp rate of 1.0°C/min. In some embodiments, the lyophilization cycle includes one or more steps having a temperature ramp rate of 2.5°C/min.

In some embodiments, each of the steps of the lyophilization cycle have a temperature ramp rate between 0.5°C/min to 3°C/min. In some embodiments, the temperature ramp rate is 0.5°C/min, 0.6°C/min ,0.7°C/min, 0.8°C/min, 0.9°C/min, 1.0°C/min, 1.1°C/min, 1.2°C/min, 1.3°C/min, 1.4°C/min, 1.5°C/min, 1.6°C/min, 1.7°C/min, 1.8°C/min, 1.9°C/min, 2.0°C/min, 2.1°C/min, 2.2°C/min, 2.3°C/min, 2.4°C/min, 2.5°C/min, 2.6°C/min, 2.7°C/min, 2.8°C/min, 2.9°C/min, or 3.0°C/min. In some embodiments, each of the steps of the lyophilization cycle have a temperature ramp rate of 1.0°C/min. In some embodiments, each of the steps of the lyophilization cycle have a temperature ramp rate of 2.5°C/min.

In some embodiments, the preserved bacterial compositions are subjected to storage conditions for a period of time following lyophilization. In some embodiments, the preserved bacterial compositions are subjected a temperature of about -100°C to -60°C, -90°C to -70°C, -95°C to -75°C, -85°C to -75°C, -85°C to -70°C, or -85°C to -65°C. In some embodiments, the preserved bacterial compositions are subjected a temperature of about -100°C, -99°C, -98°C, -97°C, -96°C, -95°C, -94°C, -93°C, -92°C, -91°C, -90°C, -89°C, -88°C, -87°C, -86°C, -85°C, -84°C, -83°C, -82°C, -81°C, -80°C, -79°C, -78°C, -77°C, -76°C, -75°C, -74°C, -73°C, -72°C, -71°C, -70°C, -69°C, -68°C, -67°C, -66°C, -65°C, -64°C, -63°C, -62°C, -61°C, or -60°C following lyophilization. In some embodiments, the preserved bacterial compositions are subjected to a temperature of about -80°C following lyophilization. In some

embodiments, the preserved bacterial compositions are subjected to a temperature of about -80°C for a period of time following lyophilization. In some embodiments, the period of time is at least 1 week, at least 2 weeks, at least 4 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year or more.

In some embodiments, the solid compositions that include bacterial strains provided herein may be formulated for administration as a pharmaceutical composition, *e.g.*, by reconstitution of a lyophilized product. The term "pharmaceutical composition" as used herein means a product that results from the mixing or combining of a solid formulation provided herein and one or more pharmaceutically acceptable excipient.

An "acceptable" excipient refers to an excipient that must be compatible with the active ingredient (e.g., the bacterial strain) and not deleterious to the subject to which it is administered. In some embodiments, the pharmaceutically acceptable excipient is selected based on the intended route of administration of the composition, for example a composition for oral or nasal administration may comprise a different pharmaceutically acceptable excipient than a composition for rectal administration. Examples of excipients include sterile water, physiological saline, solvent, a base material, an emulsifier, a suspending agent, a surfactant, a stabilizer, a flavoring agent, an aromatic, an excipient, a vehicle, a preservative, a binder, a diluent, a tonicity adjusting agent, a soothing agent, a bulking agent, a disintegrating agent, a buffer agent, a coating agent, a lubricant, a colorant, a sweetener, a thickening agent, and a solubilizer.

In one aspect, the disclosure provides compositions that allow for the preservation of bacteria. In some embodiments, the bacteria are anaerobic bacteria. Compositions useful for the preservations of bacteria are also referred to herein as stabilizing compositions. A method for preparing a preserved bacterial composition, as used herein, refers to a method that promotes the viability of the bacteria therein and allows for the recovery of the bacteria following flash freezing and lyophilizing. The stabilization or preservation functionality of the composition can be assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) at two specific time points (*e.g.*, at day 1 and at day 100). In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) at a first time point to the number of viable bacteria (*e.g.*, colony forming units) at a second time point.

In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) prior to flash freezing to the number of viable bacteria (*e.g.*, colony forming units) after flash freezing. In

some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) prior to flash freezing to the number of viable bacteria (*e.g.*, colony forming units) after lyophilizing. In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) after flash freezing/prior to lyophilizing to the number of viable bacteria (*e.g.*, colony forming units) after lyophilizing.

In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) prior to flash freezing to the number of viable bacteria (*e.g.*, colony forming units) after subjecting the composition to storage conditions for a period of time. In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) after flash freezing / prior to lyophilizing to the number of viable bacteria (*e.g.*, colony forming units) after subjecting the composition to storage conditions for a period of time. In some embodiments, the preservation functionality of the composition is assessed by comparing the number of viable bacteria (*e.g.*, colony forming units) after lyophilizing to the number of viable bacteria (*e.g.*, colony forming units) after subjecting the composition to storage conditions for a period of time.

If the number of colony forming units is the same or substantially the same at the two time points or over a time period, the composition is a perfect preserving method. A large decrease in the number of colony forming units between two time points or over a time period indicates that the method is not a good preserving composition.

In some embodiments, the methods provided herein allow for the recovery of at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 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%, up to 100% of the colony forming units over a period of time. In some embodiments, the period of time is at least 1 week, at least 2 weeks, at least 4 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year or more. In some embodiments, the percentage of recovered colony forming units or level of preservation is determined by comparing a number of colony forming units of bacteria (*e.g.,* of a bacterial strain or total bacteria) at a first time point relative to the number of colony forming units of bacteria (*e.g.*, of a bacterial strain or total bacteria) at a second time point over a period of time. For example, a 50% recovery or preservation of 50% of bacteria indicates that half of the bacteria remained viable over the

period of time; and a 100% recovery indicates that all (or substantially all) bacteria remained viable over the period of time.

In some embodiments, the methods provided herein result in a level of viability of the preserved bacterial composition of at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 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%, up to 100% of the colony forming units over a period of time. In some embodiments, the period of time is at least 1 week, at least 2 weeks, at least 4 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year or more. In some embodiments, the level of viability is determined by comparing a number of colony forming units of bacteria (*e.g.,* of a bacterial strain or total bacteria) at a first time point relative to the number of colony forming units of bacteria (*e.g.*, of a bacterial strain or total bacteria) at a second time point over a period of time. For example, a 50% viability indicates that half of the bacteria remained viable over the period of time; and a 100% viability indicates that all (or substantially all) bacteria remained viable over the period of time.

In some embodiments, the methods provided herein result in preserved bacterial compositions having enhanced viability as compared to methods involving freezing and lyophilizing bacterial compositions in freeze-drying trays (e.g., GORE® Lyoguard® freeze-drying trays. In some embodiments, the methods described herein result in preserved bacterial compositions having viability that is enhanced by at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 100-fold, 150-fold, 200-fold, 500-fold or more, as compared to methods involving freezing and lyophilizing bacterial compositions in freeze-drying trays.

SEQ ID NO:1 Strain 1 16S ribosomal RNA *Clostridium bolteae*

ATGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGAAGC
AATTAAAATGAAGTTTTCGGATGGATTTTTGATTGACTGAGTGGCGGACGGGTGAGTAACGCGTGGAT
AACCTGCCTCACACTGGGGGATAACAGTTAGAAATGACTGCTAATACCGCATAAGCGCACAGTACCGC
ATGGTACGGTGTGAAAAACTCCGGTGGTGTGAGATGGATCCGCGTCTGATTAGCCAGTTGGCGGGGTA
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GCCGCGTGAGTGAAGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTG
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AACGCGAAGAACCTTACCAAGTCTTGACATCCTCTTGACCGGCGTGTAACGGCGCCTTCCCTTCGGGG
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GAGCGCAACCCTTATCCTTAGTAGCCAGCAGGTAAAGCTGGGCACTCTAGGGAGACTGCCAGGGATAA
CCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACA
ATGGCGTAAACAAAGGGAAGCAAGACAGTGATGTGGAGCAAATCCCAAAAATAACGTCCCAGTTCGGA
CTGTAGTCTGCAACCCGACTACACGAAGCTGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCAGCAACGCCCGAAGTCAGTGA
CCCAACTCGCAAGAGAGGGAGCTGCCGAAGGCGGGGCAGGTAACTGGGGTGAAGTCGTAACAAGGTAG
CCGTATCGGAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:2 Strain 2 16S ribosomal RNA *Anaerotruncus colihominis*

TCAAAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGCGCCTAACACATGCAAGTCGAACGGAG
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AACCTGCCTTTCAGAGGGGGATAACAGCCGGAAACGGCTGCTAATACCGCATGATGTTGCGGGGGCAC
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ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGGATATTGCACAATGGGCGAAAGCCTGATGCAGCG
ACGCCGCGTGAGGGAAGACGGTCTTCGGATTGTAAACCTCTGTCTTTGGGGAAGAAAATGACGGTACC
CAAAGAGGAAGCTCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGAGCAAGCGTTGTCCG
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GTGGCTGCGTTCTAAACTGCCGTTCTTGAGTGAAGTAGAGGCAGGCGGAATTCCTAGTGTAGCGGTGA
AATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCCTGCTGGGCTTTAACTGACGCTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTACTAGG
TGTGGGGGGACTGACCCCTTCCGTGCCGCAGTTAACACAATAAGTAATCCACCTGGGGAGTACGGCCG
CAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAG
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GCATCCAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATTATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGTTGACAAAACGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCAC
TAAAACAGAGGGCGGCGACACCGCGAGGTGAAGCGAATCCCGAAAAAGTGTCTCAGTTCAGATTGCAG
GCTGCAACCCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTCGGTAACACCCGAAGCCAGTAGCCTAAC
CGCAAGGGGGGCGCTGTCGAAGGTGGGATTGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCG
GAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:3 Strain 3 16S ribosomal RNA *Ruminococcus torques*

TACGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGAAG
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CAACCTGCCTCATACAGGGGGATAACAGTTAGAAATGACTGCTAATACCGCATAAGCGCACAGGACCG
CATGGTGTAGTGTGAAAAACTCCGGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTTGGTGGGGT
AAAGGCCTACCAAGCCGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGA
CGCCGCGTGAAGGAAGAAGTATTTCGGTATGTAAACTTCTATCAGCAGGGAAGAAAATGACGGTACCT
GAGTAAGAAGCACCGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGGTGCAAGCGTTATCCGG
ATTTACTGGGTGTAAAGGGAGCGTAGACGGATAGGCAAGTCTGGAGTGAAAACCCAGGGCTCAACCCT
GGGACTGCTTTGGAAACTGCAGATCTGGAGTGCCGGAGAGGTAAGCGGAATTCCTAGTGTAGCGGTGA
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CGAGCGCAACCCTTATCTTCAGTAGCCAGCATATAAGGTGGGCACTCTGGAGAGACTGCCAGGGAGAA
CCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGGCCAGGGCTACACACGTGCTACA

ATGGCGTAAACAAAGGGAAGCGAGAGGGTGACCTGGAGCGAATCCCAAAAATAACGTCTCAGTTCGGA
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ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCAGTAACGCCCGAAGCCAGTGA
CCCAACCTTAGAGGAGGGAGCTGTCGAAGGCGGGACGGATAACTGGGGTGAAGTCGTAACAAGGTAGC
CGTATCGGAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:4 Strain 4 16S ribosomal RNA *Clostridium symbiosum*

ATGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGAAGC
GATTTAACGGAAGTTTTCGGATGGAAGTTGAATTGACTGAGTGGCGGACGGGTGAGTAACGCGTGGGT
AACCTGCCTTGTACTGGGGGACAACAGTTAGAAATGACTGCTAATACCGCATAAGCGCACAGTATCGC
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ACGGCTTACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACAC
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GCCGCGTGAGTGAAGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTG
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CGGAGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATTCTAAGTAGCCAGCGGTTCGGCCGGGAACTCTTGGGAGACTGCCAGGGATAAC
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TGGCGTAAACAAAGAGAAGCAAGACCGCGAGGTGGAGCAAATCTCAAAAATAACGTCTCAGTTCGGAC
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TACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCAGTAACGCCCGAAGTCAGTGAC
CCAACCGCAAGGAGGGAGCTGCCGAAGGCGGGACCGATAACTGGGGTGAAGTCGTAACAAGGTAGCCG
TATCGGAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:5 Strain 5 16S ribosomal RNA *Blautia producta*

ATCAGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGAA
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ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCG
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GACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCAGTAACGCCCGAAGTCAGT

GACCCAACCTTACAGGAGGGAGCTGCCGAAGGCGGGACCGATAACTGGGGTGAAGTCGTAACAAGGTA
GCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:6 Strain 6 16S ribosomal RNA *Dorea Longicatena*

AACGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGAAG
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TAACCTGCCTCATACAGGGGGATAACAGTTAGAAATGACTGCTAATACCGCATAAGACCACGGTACCG
CATGGTACAGTGGTAAAAACTCCGGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTTGGTGGGGT
AACGGCCTACCAAGCCGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCGA
CGCCGCGTGAAGGATGAAGTATTTCGGTATGTAAACTTCTATCAGCAGGGAAGAAAATGACGGTACCT
GACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTATCCGG
ATTTACTGGGTGTAAAGGGAGCGTAGACGGCACGGCAAGCCAGATGTGAAAGCCCGGGGCTCAACCCC
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ACCCAACCGTAAGGAGGGAGCTGCCGAAGGTGGGACCGATAACTGGGGTGAAGTCGTAACAAGGTAGC
CGTATCGGAAGGTGCGGCTGGATCACCTCCTTT

SEQ ID NO:7 Strain 7 16S ribosomal RNA *Erysipelotrichaceae bacterium*

ATGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCCTAATACATGCAAGTCGAACGAAG
TTTCGAGGAAGCTTGCTTCCAAAGAGACTTAGTGGCGAACGGGTGAGTAACACGTAGGTAACCTGCCC
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CCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGTCAATGGGGGAAACCCTGAACGAGCAATGC
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CCGTAAACGATGAGAACTAAGTGTTGGAGGAATTCAGTGCTGCAGTTAACGCAATAAGTTCTCCGCCT
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GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGGAAACAAATACCCTAGAGATAG
GGGGATAATTATGGATCACACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCGCATGTTACCAGCATCAAGTTGGGGACTCATGCGAGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGGCCTGGGCTACACA
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CAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATG
CTGCGGTGAATACGTTCTCGGGCCTTGTACACACCGCCCGTCAAACCATGGGAGTCAGTAATACCCGA
AGCCGGTGGCATAACCGTAAGGAGTGAGCCGTCGAAGGTAGGACCGATGACTGGGGTTAAGTCGTAAC
AAGGTATCCCTACGGGAACGTGGGGATGGATCACCTCCTTT

SEQ ID NO:8 Strain 8 16S ribosomal RNA *Subdoligranulum spp*

TATTGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGG
GTGCTCATGACGGAGGATTCGTCCAACGGATTGAGTTACCTAGTGGCGGACGGGTGAGTAACGCGTGA
GGAACCTGCCTTGGAGAGGGGAATAACACTCCGAAAGGAGTGCTAATACCGCATGATGCAGTTGGGTC
GCATGGCTCTGACTGCCAAAGATTTATCGCTCTGAGATGGCCTCGCGTCTGATTAGCTAGTAGGCGGG
GTAACGGCCCACCTAGGCGACGATCAGTAGCCGGACTGAGAGGTTGACCGGCCACATTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGC
AACGCCGCGTGAAGGAAGAAGGCTTTCGGGTTGTAAACTTCTTTTGTCGGGGACGAAACAAATGACGG
TACCCGACGAATAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGATTTACTGGGTGTAAAGGGCGTGTAGGCGGGATTGCAAGTCAGATGTGAAAACTGGGGGCTCA
ACCTCCAGCCTGCATTTGAAACTGTAGTTCTTGAGTGCTGGAGAGGCAATCGGAATTCCGTGTGTAGC
GGTGAAATGCGTAGATATACGGAGGAACACCAGTGGCGAAGGCGGATTGCTGGACAGTAACTGACGCT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATA
CTAGGTGTGGGGGGTCTGACCCCCTCCGTGCCGCAGTTAACACAATAAGTATCCCACCTGGGGAGTAC
GATCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATT
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TCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACGCAAGAGCACTCTAGCGAGACTGCCGTTGAC
AAAACGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCCTGGGCCACACACGTACTA
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GATTGCAGGCTGAAACCCGCCTGTATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTCGGGAACACCCGAAGTCCGT
AGCCTAACCGCAAGGAGGGCGCGGCCGAAGGTGGGTTCGATAATTGGGGTGAAGTCGTAACAAGGTAG
CCGTATCGGAAGGTGCGGCTGGATCACCTCCTTT

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms hall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well-known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as

described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

EXAMPLE

Studies were performed to test flash freezing liquid droplets of formulated culture before lyophilization. Testing demonstrated an improved viability of bacterial strains after freezing and lyophilizing when compared to bacterial strains that were frozen and lyophilized in freeze-drying trays (*e.g.*, GORE® Lyoguard® freeze-drying trays). Along with the improvements in viability, the ability to freeze and store frozen pellets allows greater flexibility with the production of preserved bacterial products.

Bacterial cultures were inoculated in growth media and incubated anaerobically at 37°C. Once the optical density (OD) exceeded a target threshold based on the strain to be tested, the culture was spun down and resuspended in formulation buffer. The formulation buffer used has 70g /L sucrose, 1 g/L yeast extract, 0.5 g/L L-cysteine, 20 mM histidine, and 0.1 g/L magnesium chloride. The formulation buffers for VE303-1, VE303-2 and VE303-6 also contained 0.5 g/L sodium metabisulfite. The formulation buffer used for the traditional freeze-drying method was identical, except that it did not contain magnesium chloride. The culture was spun down a second time, the supernatant discarded, and suspended again in formulation buffer. Droplets of the culture were flash frozen by adding them to a liquid nitrogen bath using a 1mL pipette. The frozen droplets were collected using a sieve and aliquoted to lyophilization vials that were on dry ice. The vials were transferred to a lyophilizer with a shelf temperature of -50°C. All vials were held at -50°C for 4 hours. Primary drying was performed at -10°C and 70 mTorr. Secondary drying was performed at +20°C and 70 mTorr. The vials were then removed from the lyophilizer and stored at -80°C until they were able to be tested.

For each bacterial strain, a harvest sample, freeze-thaw sample (data not shown), and post-lyophilization samples for each of the two methods were plated to determine viability of the bacterial strain at each stage of the process. Plates were made by producing serial dilutions for all samples in reduced phosphate buffered saline (PBS). A 100µL aliquot of the sample was mixed in 900µL of 1X PBS. Serial dilutions were performed by mixing 100µL from the previous dilution in 900µL of PBS. This was performed to generate dilutions from 10^{-1} through 10^{-7}. The 10^{-5}, 10^{-6}, and 10^{-7} dilutions were used to plate 100µL on a chocolate agar plate. The plated dilutions were spread using sterile beads and incubated anaerobically at 37°C for >48 hours. The colonies on each plates were enumerated to determine the viability of each sample. For the post-lyophilization sample, 0.1 grams of material was rehydrated in PBS prior to dilution and plating to determine viability.

The post-lyophilization viability of each bacterial strain was compared to the post-lyophilization viability of the respective bacterial strain using traditional methods of freezing bacterial culture in a freeze-drying tray (*e.g.,* GORE® Lyoguard® freeze-drying trays) (Figure 1). Bacterial strain VE303-06 demonstrated the highest improvement in viability (increased from 3.9% using a freeze-drying tray to 20% using the methods described herein).

What is claimed is:

CLAIMS

1. A method of preparing a preserved bacterial composition, comprising

flash freezing a bacterial composition, and

lyophilizing the flash frozen bacterial composition to produce a preserved bacterial composition.

2. The method of claim 1, wherein the bacterial composition comprises one or more bacterial strains.

3. The method of claim 1 or 2, wherein the bacterial composition comprises one or more anaerobic bacterial strains.

4. The method of claim 3, wherein the anaerobic bacterial strains are strict anaerobic bacteria.

5. The method of any one of claims 1-4, wherein the bacterial composition comprises one or more bacterial strains belong to the class Clostridia.

6. The method of claim 5, wherein one or more bacterial strains belong to the family Clostridiaceae.

7. The method of claim 6, wherein the bacteria comprise one or more bacterial strains belonging to the genus *Clostridium*.

8. The method of any one of claims 1-7, wherein the bacterial composition comprises one or more bacterial strains selected from the group consisting of *Clostridium bolteae, Anaerotruncus colihominis, Sellimonas intestinalis*, *Clostridium symbiosum, Blautia producta, Dorea longicatena, Clostridium innocuum,* and *Flavinofractor plautii.*

9. The method of any one of claims 1-8, wherein the bacterial composition comprises one or more bacterial strains comprising 16S rDNA sequences having at least 97% sequence

identity with the nucleic acid sequences selected from the group consisting of SEQ ID NOs: 1-8.

10. The method of any one of claims 1-9, further comprising washing the bacterial composition and resuspending the bacterial composition in a formulation buffer.

11. The method of any one of claims 1-10, wherein the flash freezing is performed by contacting the bacterial composition with a super-cooled surface.

12. The method of any one of claims 1-10, wherein the flash freezing is performed by contacting the bacterial composition with liquid nitrogen.

13. The method of any one of claims 1-12, wherein the bacterial composition has a symmetrical shape.

14. The method of claim 13, wherein the symmetrical shape is a symmetrical frozen droplet.

15. The method of any one of claims 1-14, further comprising subjecting the preserved bacterial composition to a temperature of -80°C.

16. The method of any one of claims 1-15, wherein the lyophilizing comprises a primary drying step and a secondary drying step.

17. The method of claim 16, wherein the primary drying step comprises subjecting the flash frozen bacterial composition to a temperature of -10°C and under a pressure of 70 mTorr.

18. The method of claim 16 or 17, wherein the secondary drying step comprises subjecting the flash frozen bacterial composition to a temperature of +20°C and under a pressure of 70 mTorr.

19. The method of any one of claims 1-18, further comprising culturing the bacterial composition.

20. The method of any one of claims 1-19, further comprising determining a level of viability in the preserved bacterial composition after lyophilizing.

21. The method of claim 20, wherein the level of viability in the preserved bacterial composition is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, or at least 40% of colony forming units of the bacteria over a period of time.

22. The method of claim 21, wherein the period of time is at least 1 week, at least 2 weeks, at least 4 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year or more.

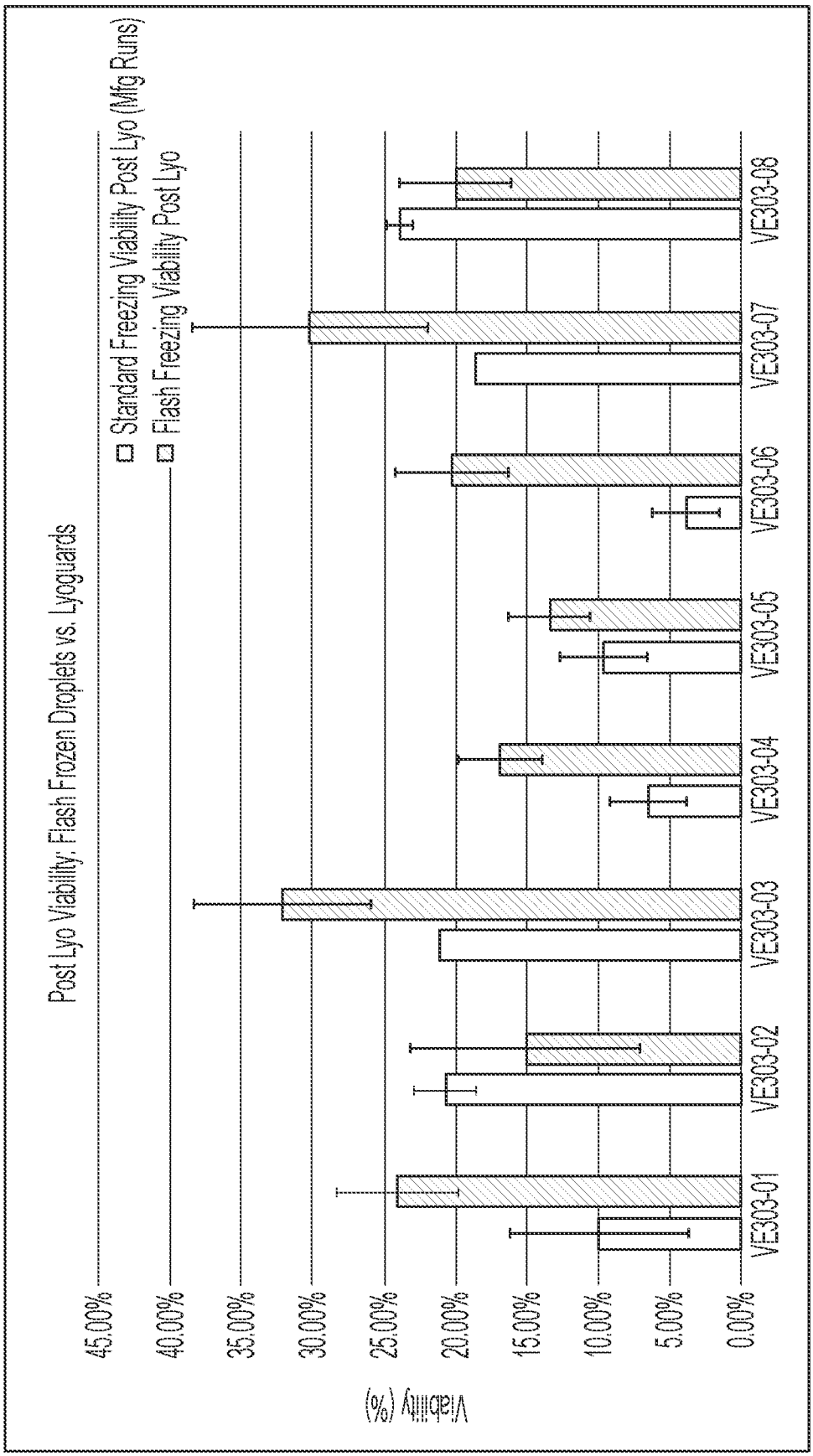

Figure 1

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/050868

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/742; A61K 35/744; A61K 45/06; C12M 1/00; C12M 1/38; C12N 1/20 (2020.01)
CPC - A61K 35/742; C12M 21/02; C12M 21/12; C12M 23/04; C12M 41/20; C12N 1/04; C12N 1/20 (2020.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/142792 A1 (ADVANCED BIONUTRITION CORPORATION) 26 September 2013 (26.09.2013) entire document	1-4
A	US 10,369,176 B2 (GOODMAN et al) 06 August 2019 (06.08.2019) entire document	1-4
A	WO 2018/112371 A1 (VEDANTA BIOSCIENCES, INC) 21 June 2018 (21.06.2018) entire document	1-4
A	WO 2018/081550 A1 (VEDANTA BIOSCIENCES, INC) 03 May 2018 (03.05.2018) entire document	1-4
P, A	WO 2020/131997 A1 (MATRISYS BIOSCIENCE, INC) 25 June 2020 (25.06.2020) entire document	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 2020	17 DEC 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/050868

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.