

PROBIOTIC-ENRICHED FRESH PRODUCE**CROSS-REFERENCE TO RELATED APPLICATIONS**

[001] This application claims the benefit of priority of Israel Patent Application No. 307266, titled "PROBIOTIC-ENRICHED FRESH PRODUCE", filed 26 September 2023, the contents of which are incorporated herein by reference in its entirety.

FIELD OF INVENTION

[002] The present invention is directed to, *inter alia*, edible products, such as fresh produce enriched with probiotic bacteria, and methods for preparing same.

BACKGROUND

[003] Due to the formation of spores, spore-forming bacteria can survive the acidic environment of the stomach and reach the intestine where they can safely propagate. An exemplary spore-forming bacterium includes the probiotic bacterium *Bacillus coagulans* which is known to produce lactic acid, and due to its excellent stability, it has been widely used in medicine, food, and chemical industry.

[004] The consumption of *B. coagulans* fosters an intestinal environment that is inhospitable to various pathogens, thereby facilitating a healthier and more efficient microbiome within the intestinal tract. This improvement in microbial balance enhances the utilization of ingested nutrients. Furthermore, *B. coagulans* amplifies the benefits of prebiotics by promoting the proliferation of beneficial bacterial populations and enhancing the production of short-chain fatty acids, which are crucial for maintaining the health of the intestinal epithelial cells. Consequently, *B. coagulans* has been demonstrated to ameliorate dysbiosis by increasing the abundance of beneficial bacterial groups.

[005] Recent studies have demonstrated that *B. coagulans* exerts therapeutic effects on various intestinal disorders, including acute diarrhea, irritable bowel syndrome (IBS), antibiotic-associated diarrhea, constipation, and inflammatory bowel disease (IBD). These effects are attributed to the modulation of microbiota composition, host immune responses, and metabolic processes.

[006] The modulation of the gut microbiota results in: a decrease of abdominal pain and bloating in subjects with IBS, reduces the number of daily bowel movements in subjects with diarrhea-predominant IBS as well as a decrease gas and bloating and improve quality of life in subjects

with post prandial intestinal gas symptoms. Additionally, *B. coagulans* has also shown anti-inflammatory and immune modulating effects.

[007] *B. coagulans* can improve the effective utilization of consumed foods, mainly due to its ability to produce a variety of enzymes. For example, a strain of *B. coagulans* has been demonstrated to secrete β -galactosidase during growth, which can degrade lactose, found in milk, into glucose and galactose, improving the digestibility of milk and effectively alleviating lactose intolerance. *In vitro* and *in vivo* studies have also found that *B. coagulans* can promote the digestion of protein and carbohydrates. Besides the production of digestive enzymes, *B. coagulans* can also generate metabolites such as diacetyl, short-chain fatty acids (SCFAs) and vitamins. *B. coagulans* was also demonstrated to promote normal intestinal peristalsis and thus, diminish the production of harmful substances allowing the improvement of the metabolic environment in the intestine. Cumulatively these effects promote healthier bowel movements and thus avoid the accumulation of toxins in the body.

[008] Furthermore, *B. coagulans* has been demonstrated to significantly enhance the health of the cells of the gut mucosal lining essentially by diminishing inflammation, which causes improved nutrient absorption capability mediated by the optimal development of the absorptive area of the villi.

[009] Recent studies have demonstrated that administration of *B. coagulans* improves the clinical outcomes of metabolic disease in morbidly obese patients undergoing laparoscopic sleeve gastrectomy. Moreover, animal studies showed that *B. coagulans* could significantly reduce the body weight, the levels of triglycerides, cholesterol, low-density lipoproteins, very low-density lipoprotein and the atherogenic index in the serum of hypercholesterolemic mice.

[010] There is still a great need for edible fresh produce, such as leafy greens, comprising and/or enriched with spore-forming bacteria, such as, but not limited to *B. coagulans*, including methods for preparing the same. Additionally, there is a need for the development of additional methodologies for the preparation of such enriched produce.

SUMMARY

[011] According to the first aspect, there is provided a fresh produce comprising a *Bacillus* strain colonizing a tissue of the fresh produce, wherein said *Bacillus* bacterial strain is *Bacillus coagulans*.

[012] According to another aspect, there is provided a fresh produce comprising a spore-forming probiotic bacterial strain, colonizing a tissue of the fresh produce, wherein at least 20% of CFU of the spore-forming bacterial strain colonizing the tissue of the fresh produce are in a spore form.

[013] According to another aspect, there is provided a kit comprising: (a) a fresh produce comprising a spore-forming bacterial strain, colonizing a tissue of the fresh produce, wherein at least 20% of CFU of the spore-forming bacterial strain colonizing the tissue of the fresh produce are in a spore form; and (b) a container configured to store the fresh produce of (a) under conditions such that: (i) at least 20% of CFU of the spore-forming bacterial strain colonizing the tissue of the fresh produce are maintained in a spore form; (ii) the fresh produce comprising the spore-forming bacterial strain is capable of improving health of a subject consuming thereof; or both (i) and (ii).

[014] According to another aspect, there is provided a method for preparing the fresh produce disclosed herein, the method comprising contacting a fresh produce with an effective amount of a composition comprising a spore-forming bacterial strain, thereby preparing the fresh produce.

[015] In some embodiments, at least 20% of colony forming unit (CFU) of the *Bacillus* strain colonizing the tissue of the fresh produce are in a spore form.

[016] In some embodiments, the *Bacillus* strain is a *B. coagulans* strain.

[017] In some embodiments, the probiotic bacterial strain is selected from the group consisting of: *B. coagulans*, *B. subtilis*, *B. indicus*, *B. lichenformis*, *B. clausii*, *B. velezensis*, and any combination thereof.

[018] In some embodiments, the *Bacillus* strain is a *B. coagulans* strain.

[019] In some embodiments, the strain is *B. coagulans* strain being selected from: BC4, BC30, or a combination thereof.

[020] In some embodiments, the fresh produce comprises at least 1×10^5 CFU of the strain per 1 gr by weight of the fresh produce.

[021] In some embodiments, the strain colonizes an intercellular space, an intracellular space, or both, of the tissue.

[022] In some embodiments, the strain constitutes at least 10% of bacteria colonizing the tissue of the fresh produce.

[023] In some embodiments, the strain is probiotic to a subject consuming thereof.

[024] In some embodiments, the strain is an exogenous endophyte of the fresh produce.

[025] In some embodiments, at least 5% of CFU of the strain survive surface disinfection of the fresh produce, thereby determined as exogenous endophyte of the fresh produce.

[026] In some embodiments, the fresh produce is a processed product.

[027] In some embodiments, the processed product is cut, sterilized, washed, or any combination thereof.

[028] In some embodiments, the sterilized product underwent surface sterilization. In some embodiments, the sterilized product is a surface-sterilized product.

[029] In some embodiments, the strain is exogenously applied to the fresh produce pre-harvest, post-harvest, or both.

[030] In some embodiments, the fresh produce is selected from the group consisting of: a plant or plant material, a fungus or fungus material, and any combination thereof.

[031] In some embodiments, the plant or plant material comprises any one of: leaf, fruit, root, tuber, bulb, stem, seed, and any combination thereof.

[032] In some embodiments, the fresh produce is a leafy green.

[033] In some embodiments, the fresh produce is for use in prevention or treatment of dysbiosis in a subject in need thereof.

[034] In some embodiments, the composition comprises at least one agent capable of maintaining at least 20% of CFU of the spore-forming bacterial strain of the composition in a spore form.

[035] In some embodiments, the agent is selected from the group consisting of: surfactant, pH adjuster, an oil, and a combination thereof.

[036] In some embodiments, the spore-forming bacterial strain is capable of improving health of a subject consuming thereof.

[037] In some embodiments, the container is further configured to store the fresh produce under conditions such that the fresh produce comprising the spore-forming bacterial strain is capable of improving health of a subject consuming thereof.

[038] In some embodiments, the contacting is pre-harvest contacting, post-harvest contacting, or both.

[039] In some embodiments, the contacting is post-harvest contacting, and the method further comprises a step before the contacting comprising disinfecting or sterilizing the fresh produce.

[040] In some embodiments, the sterilizing comprises sterilizing a surface of said fresh produce.

[041] In some embodiments, the contacting is spraying.

[042] In some embodiments, the composition is formulated for spraying.

[043] Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

[044] Further embodiments and the full scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[045] **Fig. 1** includes a photomicrograph showing *Bacillus coagulans*, obtained from the inner tissue of lettuce comprising this spore-forming probiotic bacterial strain colonizing the tissue of this fresh produce (after surface disinfection and plant tissue crushing). The detection was performed under a light microscope. Most bacteria are in the spore production stage (arrowheads).

[046] **Figs. 2A-2C** include graphs showing endophytic level of BC4 as a function of % spore in inoculation culture in lettuce (**2A**), mushrooms (**2B**), and carrots (**2C**).

[047] **Figs. 3A-3B** include graphs showing total survival (**3A**) and endophytic survival (**3B**) of 0%, 25%, 50%, 75%, or 100% *B. coagulans* BC4 in endospore form complemented to 100% with vegetative *B. coagulans* BC4, in lettuce.

[048] **Figs. 4A-4B** include graphs showing total survival (**4A**) and endophytic survival (**4B**) of 0%, 25%, 50%, 75%, or 100% *B. coagulans* BC4 in endospore form complemented to 100% with vegetative *B. coagulans* BC4, in carrots.

[049] **Figs. 5A-5B** include graphs showing total survival (**5A**) and endophytic survival (**5B**) of 0%, 25%, 50%, 75%, or 100% *B. coagulans* BC4 in endospore form complemented to 100% with vegetative *B. coagulans* BC4, in mushrooms.

[050] **Figs. 6A-6B** include graphs showing total survival and endophytic survival of *B. coagulans* BC4 subterminal spores or free spores in lettuce (**6A**) and baby carrots (**6B**).

DETAILED DESCRIPTION

Fresh produce

[051] According to one aspect, there is provided a fresh produce comprising a bacterial strain colonizing a tissue thereof.

[052] According to another aspect, there is provided a fresh produce comprising a spore-forming bacterial strain colonizing a tissue thereof.

[053] According to another aspect, there is provided a disinfected fresh produce comprising an exogenous spore-forming bacterial strain colonizing a tissue thereof.

[054] In some embodiments, the fresh produce is disinfected prior to being exogenously applied with the spore-forming bacterial strain. In some embodiments, the spore-forming bacterial strain colonizes the fresh produce after being disinfected. In some embodiments, the spore-forming bacterial strain is an exogenous, non-natural, non-endogenous, endophyte of the fresh produce, or any combination thereof. In some embodiments, the strain is an exogenous endophyte of the fresh produce. In some embodiments, the strain is not an epiphyte. In some embodiments, an endophyte survives sterilization of the fresh produce. In some embodiments, an endophyte survives surface sterilization of the fresh produce. In some embodiments, an endophyte withstands sterilization of the fresh produce or a surface thereof. In some embodiments, an endophyte maintains the ability to proliferate in a tissue of the fresh produce following sterilization thereof.

[055] In some embodiments, the *Bacillus* bacterial strain comprises or is *Bacillus coagulans*.

[056] In some embodiments, a strain as used herein is a bacterial strain.

[057] In some embodiments, at least 20% of colony forming unit (CFU), at least 30% of CFU, at least 40% of CFU, at least 50% of CFU, at least 60% of CFU, at least 70% of CFU, at least 80% of CFU, at least 90% of CFU, at least 95% of CFU, at least 97% of CFU, at least 99% of CFU, or any value and range therebetween, of the strain (such as spore-forming *Bacillus* strain) colonizing a tissue of the fresh produce are in a spore form.

[058] Means and methods for identifying, determining, quantifying, and any combination thereof, of bacterial spore(s) are common and would be apparent to one of ordinary skill in the art. Non-limiting example for a method of determining the presence of bacterial spores includes, but is not limited to, light microscopy, as exemplified herein.

[059] In some embodiments, a strain is selected from: *B. coagulans*, *B. subtilis*, *B. indicus*, *B. licheniformis*, *B. clausii*, *B. velezensis*, or any combination thereof.

[060] In some embodiments, a strain comprises or is *B. coagulans*.

[061] In some embodiments, a *B. coagulans* strain is selected from: BC4, BC30, or a combination thereof.

[062] As used herein, *B. coagulans* strain BC4 is also known as “*B. coagulans* SNZ 1969®”. As used herein, *B. coagulans* BC30 is also known as “BC^{30TM}”, “Ganeden BC30®, *Bacillus coagulans* BC30TM, or the like”.

[063] In some embodiments, the fresh produce comprises at least 1×10^4 CFU, at least 1×10^5 CFU, at least 1×10^6 CFU, at least 1×10^7 CFU, at least 1×10^8 CFU, at least 1×10^9 CFU, at least 1×10^{10} CFU, or any value and range therebetween, of the strain or bacterial strain per 1 gr by weight of the fresh produce. Each possibility represents a separate embodiment of the invention.

[064] In some embodiments, the strain colonizes an intercellular space of the tissue. In some embodiments, the strain colonizes an intracellular space of the tissue. In some embodiments, the strain colonizes an intercellular space and an intracellular space of the tissue.

[065] In some embodiments, the strain constitutes at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of bacteria colonizing the tissue of a fresh produce, or any value and range therebetween. In some embodiments, the strain constitutes at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of probiotic bacteria colonizing the tissue of a fresh produce, or any value and range therebetween. Each possibility represents a separate embodiment of the invention. In some embodiments, the strain constitutes at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of the endophytic bacteria colonizing the tissue of a fresh produce, or any value and range therebetween. In some embodiments, the strain constitutes at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of probiotic endophytic bacteria colonizing the tissue of a fresh produce, or any value and range therebetween. Each possibility represents a separate embodiment of the invention.

[066] In some embodiments, the strain is probiotic to a subject consuming thereof.

[067] As used herein, the terms “probiotic”, “probiotic strain”, and “probiotic bacterial strain” are interchangeable, and encompass any bacterial strain having at least one positive effect or health

benefit when consumed or administered to a subject. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human subject.

[068] In some embodiments, at least 1%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of CFU of the strain survive surface disinfection of the fresh produce, or any value and range therebetween, thereby determined as exogenous endophyte of the fresh produce. Each possibility represents a separate embodiment of the invention.

[069] Means and methods for surface sterilization, such as of plant material, including fresh produce, are common and would be apparent to one of ordinary skill in the art. Non-limiting examples include, but are not limited to, spraying, washing, fogging, fumigating, or any combination and/or equivalent thereof, with a disinfecting material and/or solution, such as exemplified herein, using bleach, ethanol, or their combination.

[070] In some embodiments, the fresh produce of the invention, e.g., comprising the strain colonizing a tissue thereof, is a processed product. In some embodiments, processed comprises minimally processed.

[071] In some embodiments, the fresh produce of the invention, e.g., comprising the strain colonizing a tissue thereof, is a fresh cut product.

[072] In some embodiments, a processed product or minimally processed is cut, sterilized, washed, chilled, frozen, or any combination thereof.

[073] In some embodiments, frozen comprises snap frozen, deep frozen, or the like.

[074] In some embodiments, the fresh produce is devoid of epiphytic bacteria upon sterilization. In some embodiments, an epiphyte does not survive sterilization as disclosed herein.

[075] In some embodiments, a sterilized product undergoes or undergone surface sterilization. In some embodiments, sterilization comprises chemical sterilization or sterilization with a chemical compound. In some embodiments, sterilization is with bleach, ethanol, or a combination thereof.

[076] In some embodiments, the strain is exogenously applied to the fresh produce pre-harvest. In some embodiments, the strain is exogenously applied to the fresh produce post-harvest. In some embodiments, the strain is exogenously applied to the fresh produce pre-harvest and post-harvest.

[077] In some embodiments, the fresh produce is for use in prevention or treatment of dysbiosis in a subject in need thereof.

[078] In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human subject.

[079] In some embodiments, the subject is afflicted with or having predisposition to developing dysbiosis.

[080] As used herein, the term “dysbiosis” refers to imbalance in a microbial population of a subject, such as gut microbiota. In some embodiments, dysbiosis is associated with a disease or a disorder. In some embodiments, dysbiosis is a disease or a disorder. In some embodiments, dysbiosis comprises or is gut dysbiosis.

[081] In some embodiments, the fresh produce of the invention is stable (or more stable than a control). In some embodiments, the fresh produce of the invention has increased stability compared to a control. In some embodiments, the fresh produce of the invention has increased shelf-life compared to a control. In some embodiments, increased stability, increased shelf-life, or both, relate to the decaying or decay of the fresh produce. In some embodiments, increased stability, increased shelf-life, or both, compared to a control is determined at conventional conditions of fresh produce storage. In some embodiments, conventional conditions of fresh produce storage are at a temperature ranging from 2 °C to 30 °C, 2 °C to 20 °C, 2 °C to 15 °C, 2 °C to 10 °C, 2 °C to 8 °C, 4 °C to 10 °C, 3 °C to 12 °C, or 4 °C to 6 °C. Each possibility represents a separate embodiment of the invention.

[082] In some embodiments, the fresh produce comprising the spore-forming bacterial strain, wherein at least 20% of CFU of the bacterial strain are in a spore form, are less prone to decay, decay less, decay slower, or any combination thereof, compared to a control.

[083] In some embodiments, a control comprises a fresh produce being devoid of the strain or bacterial strain as disclosed herein. In some embodiments, a control comprises a fresh produce comprising no spores of the spore-forming bacterial strain as disclosed herein. In some embodiments, a control comprises a fresh produce being devoid of spores of the spore-forming bacterial strain as disclosed herein. In some embodiments, a control comprises a fresh produce comprising the spore-forming bacterial strain as disclosed herein, wherein not more the 1%, not more the 2%, not more the 5%, not more the 7%, not more the 10%, not more the 15%, not more the 19% of the CFU of the disclosed spore-forming bacterial strain are in the form of a spore.

[084] In some embodiments, a fresh produce comprises or is a plant fresh produce. In some embodiments, a fresh produce comprises or is a fungus fresh produce.

[085] Furthermore, a plant fresh produce (including fruit, vegetables, tubers, bulbs, and leafy produce) are the most important raw-eaten food worldwide and are a substantial part of a balanced and healthy diet. Many beneficial effects on health and lifestyle are attributed to the consumption of fresh-cut or minimally processed edible plant products as they represent a good source of

vitamins, e.g., b-carotene, ascorbic acid, riboflavin, and folic acid as well as minerals, e.g., iron, calcium, manganese and phosphorous. Moreover, plant fresh produce is known to be rich sources of micronutrients as well as dietary fiber, particularly in leafy produce, enabling the intake of these nutrients. Finally, the relatively low amount of carbohydrates and fats, particularly in vegetables and leafy foods correlates with a low-calorie value, making them leading candidates for healthy diet. In addition, the presence of non-digestible or low-digestible food ingredient characterizes plant fresh produce as prebiotic substance, stimulating the growth or activity of beneficial probiotic bacteria in the human colon (Biedrzycka B., Polish J. food Nutr. Sci. 13, 143-150, 2004).

[086] In some embodiments, a fresh produce is selected from: a plant or plant material, a fungus or fungus material, and any combination thereof.

[087] In some embodiments, a plant or plant material comprises any one of: leaf, fruit, root, tuber, bulb, stem, seed, and any combination thereof.

[088] In some embodiments, the fresh produce comprises or is a leafy green.

[089] In some embodiments, the fresh produce further comprises a metabolite derived from the bacterial strain. In some embodiments, the fresh produce is enriched with the metabolite. In some embodiments, the fresh produce comprises the metabolite in an amount, abundance, or both, being greater than that of a control fresh produce. In some embodiments, a control fresh produce is devoid of the bacterial strain as disclosed herein. In some embodiments, the metabolite maintains or improves health, wellbeing, or both, of a subject consuming same. In some embodiments, the metabolite comprises a primary metabolite, a secondary metabolite, or a combination thereof. In some embodiments, the metabolite comprises a plurality of types of metabolites.

[090] As used herein, the term "plurality" refers to any integer being equal to or greater than 2.

A kit

[091] According to another aspect, there is provided a kit comprising: (a) a fresh produce comprising a spore-forming bacterial strain, colonizing a tissue of the fresh produce; and (b) a container configured to store the fresh produce of (a).

[092] In some embodiments according to the kit of the invention, at least 20% of CFU of the spore-forming bacterial strain colonizing the tissue of the fresh produce are in a spore form.

[093] In some embodiments, the container is configured to store the fresh produce under conditions such that: (i) at least 20% of CFU of the spore-forming bacterial strain colonizing the tissue of the fresh produce are maintained in a spore form; (ii) the fresh produce comprising the

spore-forming bacterial strain is capable of improving health of a subject consuming thereof; or both (i) and (ii).

[094] In some embodiments, the kit is for maintaining or increasing stability of the fresh produce comprising the bacterial strain. In some embodiments, the kit is for increasing shelf-life of the fresh produce comprising the bacterial strain.

Method of preparation

[095] According to another aspect, there is provided a method for preparing the fresh produce of the invention. In some embodiments, the method is for preparing a fresh produce comprising a probiotic bacterial strain, and wherein the fresh produce is characterized by increased stability, shelf-life, or both, compared to a control.

[096] In some embodiments, the method comprises contacting a fresh produce with an effective amount of a composition comprising a spore-forming bacterial strain, thereby preparing the fresh produce of the invention.

[097] In some embodiments, the composition comprises further at least one agent selected from: surfactant, pH adjuster, an oil, or a combination thereof.

[098] In some embodiments, the oil is or comprises sunflower oil.

[099] In some embodiments, the agent is capable of maintaining at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of CFU of the spore-forming bacterial strain of the composition in a spore form, or any value and range therebetween. Each possibility represents a separate embodiment of the invention.

[0100] In some embodiments, the composition comprises at least one agent capable of maintaining at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of CFU of the spore-forming bacterial strain of the composition in a spore form, or any value and range therebetween. Each possibility represents a separate embodiment of the invention.

[0101] In some embodiments, the agent is selected from: surfactant, pH adjuster, or a combination thereof.

[0102] In some embodiments, the contacting is pre-harvest contacting. In some embodiments, the contacting is post-harvest contacting. In some embodiments, the contacting is pre-harvest contacting and post-harvest contacting.

[0103] In some embodiments, the method further comprises a step before the contacting comprising disinfecting or sterilizing the fresh produce.

[0104] In some embodiments, the contacting is post-harvest contacting, and the method further comprises a step before the contacting comprising disinfecting or sterilizing the fresh produce.

[0105] In some embodiments, the fresh produce comprises no bacteria or is devoid of bacteria after sterilization and before being inoculated and/or colonized by the bacterial strain or strain as disclosed herein.

[0106] In some embodiments, sterilizing comprises sterilizing a surface of the fresh produce.

[0107] In some embodiments, sterilizing comprises reducing the amount, concentration, abundance, or any combination thereof of microbes, including, but not limited to bacteria, on the fresh produce. In some embodiments, sterilizing need not mean full or complete eradication of all microbes.

[0108] In some embodiments, contacting is spraying.

[0109] In some embodiments, the composition is formulated for spraying. In some embodiments, the composition further comprises an agriculturally acceptable carrier. In some embodiments, the composition is an agricultural composition.

[0110] In some embodiments, the composition is devoid of one or more compounds capable of or required for activation of a spore into an active bacteria. In some embodiments, the composition comprises one or more compounds capable of or required for active bacterial proliferation, survival, or both, and is being devoid of one or more compounds capable of or required for activation of a spore into an active bacteria. In some embodiments, the composition is suitable for bacteria proliferation, survival, or both. In some embodiments, the composition is suitable for preserving, maintaining, or both, bacterial spores in a spore form. In some embodiments, composition is suitable for production, secretion, accumulation, or any combination thereof, of a metabolite by the bacterial strain. In some embodiments, the metabolite maintains or improves health, wellbeing, or both, of a subject consuming same.

[0111] As used herein, the terms “treatment” or “treating” of an infection, disease, disorder, or condition, encompasses alleviation of at least one symptom thereof, a reduction in the severity thereof, or inhibition of the progression thereof. Treatment need not mean that the infection, disease, disorder, or condition is totally cured. To be an effective treatment, a useful composition herein needs only to reduce the severity of an infection, disease, disorder, or condition, reduce the severity of symptoms associated therewith, or provide improvement to a subject’s quality of life.

[0112] As used herein, the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical

symptoms of a condition. In some embodiments, treating comprises preventing. In some embodiments, treating comprises preventing and treating.

[0113] In some embodiments, there is provide a method of treating or preventing dysbiosis in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the fresh produce of the invention.

[0114] In some embodiments, administering comprises feeding the subject on the fresh produce.

General

[0115] As used herein, the term "about" when combined with a value refers to plus and minus 10% of the reference value. For example, a length of about 1,000 nanometers (nm) refers to a length of 1,000 nm $\pm$ 100 nm.

[0116] It is noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polynucleotide" includes a plurality of such polynucleotides and reference to "the polypeptide" includes reference to one or more polypeptides and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only", and the like in connection with the recitation of claim elements or use of a "negative" limitation.

[0117] In those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., "a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0118] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the invention are specifically

embraced by the present invention and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0119] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

[0120] Various embodiments and aspects of the present invention as delineated herein above and as claimed in the claims section below find experimental support in the following examples. Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non-limiting fashion.

[0121] Generally, the nomenclature used herein, and the laboratory procedures utilized in the present invention include chemical, molecular, biochemical, and cell biology techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); The Organic Chemistry of Biological Pathways by John McMurry and Tadhg Begley (Roberts and Company, 2005); Organic Chemistry of Enzyme-Catalyzed Reactions by Richard Silverman (Academic Press, 2002); Organic Chemistry (6th Edition) by Leroy "Skip" G Wade; Organic Chemistry by T. W. Graham Solomons and, Craig Fryhle.

EXAMPLE 1

Materials and Methods

Pre-harvest application

Plant materials

[0122] Seven (7) lettuce varieties were used for these experiments: 3 Butterheads (Sartre, Gauguin and DaVinci); 3 Romaines (Cerventes, Noga936 and Lior); and one Iceberg (Liad). The screening

was performed on 4-week-old lettuce plantlets. The lettuces were grown at 25 °C in a plant growth chamber (MRC) and harvested 6 days post application.

Probiotic inoculation

[0123] The application was made by spraying the plantlets with an inoculation solution of a freeze-dried single strain of probiotic (See **Table 1**) diluted in sterile 2% CMC (carboxymethyl cellulose) solution. The application volume was approx. 1.5 ml per lettuce plantlet. The inoculation solutions contained 10^7 - 10^8 CFU/ml, as was verified by plating on selective agar (See **Table 1**). Each combination was applied in 4-6 replicates.

Surface disinfection to remove epiphytic bacteria

[0124] Plantlets were subjected to surface disinfection through immersion in a 0.3% bleach solution for 60 seconds, followed by a 10-second exposure to 70% Ethanol, and subsequently, underwent three rinses with microbially UV-filtered water (FW).

Live count analysis of endophytic probiotics

[0125] Three (3) plantlets were considered as one replicate. Each replicate was weighed and then crushed for 2 minutes in 50 ml of FW using BagMixer 400S (Interscience) in a crushing bag with an internal filter membrane (BagFilter 400 P @sterile@ bag with side filter). The filtered extract was gathered from the bag, diluted 10-fold and 100-fold, and quantified using drop plating (plating three 50 microliter droplets on a selective media agar) for 48 hours at 37 °C. After the incubation, colonies were visually identified and counted, and colony PCR was performed to validate the probiotic strain using specific PCR primers. CFU/gr (colony forming units per 1 gr of lettuce) was calculated considering the three droplets' colony number average, the droplet volume, the two extract dilutions, the colony number average, the total extraction volume, and the extracted lettuce weight.

Post-harvest application

Plant materials

[0126] This experiment used two lettuce varieties: Sartre and Gauguin Sartre. The lettuces were grown in a nutrient film technique (NFT) hydroponic system in the Galilee region in northern Israel, and were harvested 24-48 hours before the experiment.

Plant preparations and process

[0127] The leaves were separated from the lettuce head main stem and cut using a kitchen knife to approximately 4 cm × 4 cm pieces. Fresh-cut leaves were then disinfected twice using diluted bleach (100 ppm of Cl^- for one minute). This kind of disinfection is common in the fresh-cut lettuce

industry and is part of the process. Fresh-cut leaves obtained from one lettuce were considered as one replicate.

Probiotic inoculation

[0128] Twenty-two combinations were examined in addition to negative control (lettuce without bacteria application). Each combination was composed of an inoculation solution of a freeze-dried single strain of probiotic (See **Table 1**) diluted in microbially UV-filtered water (FW) and applied by spraying and then resting for 30 min at room temperature on a lettuce variety. The Inoculation solution volume was 5 ml per lettuce head. The inoculation solutions contained 10^7 - 10^8 CFU/ml, as was verified by plating on selective agar (See **Table 1**). Each combination was applied in 12 replicates.

Surface disinfection to remove epiphytic bacteria

[0129] Four (4) replicas of each combination were disinfected by different methods, as described in **Table 2**. After the disinfection, the plants were washed three times in FW.

Live count analysis of endophytic probiotics

[0130] Approximately 50% (20-40 gr) of each replicate was weighed and then crushed for 2 minutes in 50 ml of FW using BagMixer 400S (Interscience) in a crushing bag with an internal filter membrane (BagFilter 400 P @sterile@ bag with side filter). The calculation for the colonies' number was performed as described above.

Table 1. Probiotic and non-probiotic bacterial strains used in the current study

Probiotic strain	Strain code	Selective media
<i>Lactobacillus plantarum FLORAFIT KP-LP-115</i>	106	Chromagar + MRS
<i>Lactobacillus paracasei FLORAFIT KP-Lpc®</i>	107	Chromagar + MRS
<i>Lactobacillus acidophilus NCFM Danisco</i>	112	Chromagar + MRS
<i>Lactobacillus rhamnosus GG DELVOPRO GG DSL</i>	102	Chromagar + MRS
<i>Lactobacillus rhamnosus HN001 NZMP LactoB Fonterra</i>	110	Chromagar + MRS

<i>Bifidobacterium lactis</i> HN019™ Danisco	111	MRS agar (anaerobic)
<i>Bifidobacterium lactis</i> FLORAFIT KP-BI-04®	108	MRS agar (anaerobic)
<i>Bacillus coagulans</i> BC4 Sacco	109	Chromagar + MRS agar
<i>Bacillus coagulans</i> BC30 Kerry	113	Chromagar + MRS agar
<i>Bacillus cereus</i>	<i>B. cereus</i>	Nutrient agar
<i>Frateuria defendens</i>		CV agar
Negative control	N.C	Chromagar + MRS agar

Table 2. Surface disinfection protocols that were used in post-harvest experiment

Surface Disinfection	Bleach	Ethanol
Mild disinfection	0.3% for 60 sec	70% for 10 sec
Strong disinfection	3% for 60 sec	70% for 60 sec
No disinfection	None	None

Results

[0131] The inventors found that interaction between plant variety and probiotic bacteria six days after spray application of pre-harvest lettuce, were strain specific. In that regard, surprisingly, both *Bacillus coagulans* strains were found to inhabit or colonize any one of the examined lettuce varieties, and with high frequency.

[0132] Further, the inventors examined the ability of probiotic bacteria to colonize post-harvested plant varieties. The results show that similarly to pre-harvest treatment, the spore-forming *Bacillus* exhibited true endophytic activity, e.g., colonized inner plant tissue- hence were protected from harsh surface disinfection (or removal by sonication). Specifically of the spore-forming *Bacillus* species, tissue colonization of *B. coagulans* was found to be even greater than that of *B. cereus*,

and especially the *B. coagulans* BC4 strain showed increased endophytic quantity (or colonization and survival of disinfection) in all of the examined lettuce varieties under both pre- and post-harvest applications.

[0133] Further to the above, the inventors have shown that *Bacillus coagulans* that was obtained from the inner tissue of lettuce (after surface disinfection and plant tissue crushing), previously been applied with the bacteria, as described herein, was primarily in the spore production stage (**Fig. 1**).

[0134] All in all, the inventors conclude that in both pre- and post-harvest applications in leafy green, such as lettuce, spore-forming bacteria, such as *Bacillus coagulans* exhibited superior penetration and survival rates during surface sterilization compared to other probiotic strains. Thus, spore-forming *Bacillus*, and specifically, *B. coagulans*, is suggested for colonization of fresh produce, including leafy green products (such as lettuce) e.g., for the production of endophytic probiotic-enriched plant material.

Post-harvest application

[0135] Further to the above, the inventors have examined post-harvest inoculation of mushrooms with spore-forming bacteria, compared to a non-spore forming bacteria.

[0136] The post-harvest treatment included inoculation with *B. coagulans* strain BC4 and *L. plantarum*. The probiotic concentration in the inoculation solution was $\sim 10^7$ CFU/ml.

[0137] Inoculation was performed using three different methods: (i) Spraying (extraction after 3 hours – mild disinfection); (ii) Injection to the stipe; or (iii) injection to the cap (extraction after 18 hours – no disinfection). Two doses of each injection method were applied. Each experiment was performed in two replicated.

Results

[0138] For both probiotics no significant differences were observed between the different inoculation methods.

[0139] Importantly, *B. coagulans* strain BC4 was found to be significantly better compared to *L. plantarum* and reached as many as 10^6 CFU/gr mushroom.

[0140] Thus, spore-forming *Bacillus*, and specifically, *B. coagulans*, is suggested for colonization of fungal products (such as mushrooms) e.g., for the production of endophytic probiotic-enriched plant material.

[0141] All in all, the inventors showed advantage of spore probiotics over non-spore probiotic strains and over spore and non-spore endophytic bacteria in different crops, including: lettuce,

baby carrot, mushrooms, pepper, cherry tomato, blueberries, baby cucumber, sunflower, pea, parsley, coriander, and thyme.

EXAMPLE 2

[0142] Further, the inventors showed that probiotic bacterial strain in the form of endospores has better penetration and survival compared to the vegetative form of the same strain, for example, in lettuce.

Penetration

[0143] The inventors demonstrated that 100% BC4 spores inoculate (e.g., a composition wherein 100% of BC4 cells are in a spore form) had a significantly higher penetration rate and CFU/gr of endophytic probiotic enrichment, compared to 100% vegetative BC4 inoculate (e.g., a composition wherein 100% of BC4 cells are in a vegetative [non-spore] form). The different treatments were tested immediately after 30 minutes of inoculation.

Survival

Tested 24 hr after 30 minutes inoculation

[0144] The inventors have demonstrated that around 60% of the BC4 spores applied on the lettuce survived on the lettuce. Out of this 60%, about 10% of the cells were endophytic (6%). In sharp contrast, only 5% of the BC4 in the vegetative form that was applied on the lettuce survived on the lettuce. Out of this 5%, less than 2% of the cells were endophytic (0.1%- 0.5%).

[0145] The application of the BC4 inoculation culture, comprising a mixture of spores and vegetative cells in defined percentages, exhibited non-linear behavior. The system produced a saturation curve, demonstrating enhanced penetration and survival when the spore concentration exceeded 25%. This was demonstrated in lettuce, mushrooms, and carrots (**Figs. 2A-2C**, respectively).

Tested 11 days after 30 minutes inoculation (end of shelf life)

[0146] The inventors have demonstrated that for 100% spore BC4 inoculate no significant change in total and endophytic CFU/gr was observed over the examined period. In sharp contrast, in the case of 100% vegetative BC4 inoculate, 100% mortality was observed. In mixed inoculate of 25% spore, slight to no decrease in the total and endophytic CFU/gr was observed.

EXAMPLE 3

[0147] The inventors have further screened and/or compared penetration and survival of probiotic bacterial strains having: vegetative form, subterminal spore form, and free endospore form.

[0148] The probiotic strains used in the screening, including their spore type/form, and size (e.g., length), are listed in **Table 3**. All strains are characterized by/known to present a non-significant random movement. The results are summarized in **Tables 4-6** for lettuce, carrots, and mushrooms, respectively.

Table 3. Probiotic strains used in the current analysis

<i>Strain</i>	Form	Rep.	length [μm]
<i>Bacillus coagulans BC4</i>	subterminal endospores	6	2.23
<i>Bacillus coagulans BC4</i>	free endospores	6	0.56
<i>Bacillus coagulans BC4</i>	vegetative	6	0.74
<i>Bacillus coagulans BC30</i>	subterminal endospores	6	2.25
<i>Bacillus coagulans BC01</i>	subterminal endospores	6	1.79
<i>Bacillus coagulans BC01</i>	free endospores	6	0.61
<i>Bacillus subtilis Bss-19</i>	free endospores	6	0.92
<i>Bacillus subtilis HU58</i>	free endospores	6	0.80
<i>Bacillus subtilis DE111</i>	free endospores	6	0.80
<i>Bacillus subtilis MB40</i>	free endospores	6	0.24
<i>Lactobacillus plantarum</i>	vegetative	6	0.70
<i>Lactobacillus paracasei</i>	vegetative	6	0.61
<i>Lactobacillus rhamnosus HN001</i>	vegetative	6	0.53
<i>Lactobacillus acidophilus</i>	vegetative	6	0.58

Table 4. Results in inoculated lettuce

Protocol	24 h				Middle (4-6 days)				End of shelf-life (11 days)			
	Probiotic enrichment				Probiotic enrichment				Probiotic enrichment			
	Total P-CFU/gr	Efficiency	Enriched reps		Total P-CFU/gr	Efficiency	Enriched reps		Total P-CFU/gr	Efficiency	Enriched reps	
Total CFU												
1.86E+08	1.21E+05 A	6.51% A	4/4		1.05E+05	5.65%	4/4		1.47E+05	7.89% A	4/4	
2.94E+08	1.05E+05 AB	3.57% B	4/4		1.12E+05	3.82%	4/4		1.58E+05	5.39% AB	4/4	
3.02E+08	**1.03E+05 AB	3.41% B	4/4		*5.17E+04	1.71%	4/4		*5.10E+04	1.69% B	4/4	
4.99E+08	1.14E+05 AB	2.29% BC	4/4		9.94E+04	1.99%	4/4		1.02E+05	2.03% B	4/4	
5.56E+08	7.84E+04 AB	1.41% C	4/4		2.04E+05	3.67%	4/4		7.28E+04	1.31% B	4/4	
4.73E+08	1.03E+05 AB	2.17% BC	4/4		1.32E+05	2.79%	4/4		1.46E+05	3.08 AB	4/4	
4.33E+08	5.12E+04 AB	1.18% C	4/4		5.16E+04	1.19%	4/4		1.03E+05	2.38% B	4/4	
1.86E+08	0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
1.84E+09	0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
3.28E+08	0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
4.75E+08	0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
-	0.00E+00	-	0/4		0.00E+00	-	0/4		0.00E+00	-	0/2	

Treatment	Probiotic	Dilution	Disinfection	CFU/ml inoculation solution	Volume (ml/100 gr)
1	<i>Bacillus coagulans BC4</i>	1:1,000	Long	3.72E+07	5
2	<i>Bacillus coagulans BC30</i>	1:100	Long	5.88E+07	5
3	<i>Bacillus coagulans BC01</i>	1:2,000	Long	6.03E+07	5
4	<i>Bacillus subtilis Bss-19</i>	1:2,000	Long	9.98E+07	5
5	<i>Bacillus subtilis HU58</i>	1:1,000	Long	1.11E+08	5
6	<i>Bacillus subtilis DE111</i>	1:1,000	Long	9.47E+07	5
7	<i>Bacillus subtilis MB40</i>	1:1,000	Long	8.67E+07	5
8	<i>Lactobacillus plantarum</i>	1:10,000	Long	3.72E+07	5
9	<i>Lactobacillus paracasei</i>	1:1,000	Long	3.68E+08	5
10	<i>L. rhamnosus HN001</i>	1:10,000	Long	6.57E+07	5
11	<i>Lactobacillus acidophilus</i>	1:2,000	Long	9.50E+07	5
12	N.C	-	-	-	-

Table 5. Results in inoculated carrots

24 h				Middle (4-6 days)				End of shelf-life (11 days)			
Probiotic enrichment				Probiotic enrichment				Probiotic enrichment			
Total P-CFU/gr	Efficiency	Enriched reps		Total P-CFU/gr	Efficiency	Enriched reps		Total P-CFU/gr	Efficiency	Enriched reps	
*3.60E+05 A	10.73% A	4/4		-	-	-		**7.49E+05 AB	22.35% AB	4/4	
3.37E+05 A	14.72% A	4/4		-	-	-		4.68E+05 B	20.43% AB	4/4	
*8.16E+04 B	1.85% B	4/4		-	-	-		**3.58E+05 B	8.13% B	4/4	
*3.68E+05 A	12.32% A	4/4		-	-	-		**7.67E+05 AB	25.70% AB	4/4	
*3.01E+05 A	8.88% A	4/4		-	-	-		**9.48E+05 AB	27.95% AB	4/4	
*4.69E+05 A	13.84% A	4/4		-	-	-		**1.25E+06 A	36.73% A	4/4	
*8.29E+04 B	2.37% B	2/4		-	-	-		**7.59E+05 AB	21.68% AB	4/4	
8.12E+03 B	0.24% B	2/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/4		0.00E+00	0.00%	0/2	
0.00E+00	-	0/4		0.00E+00	-	0/4		0.00E+00	-	0/4	

Protocol						
Treatment	Probiotic	Dilution	Disinfection	CFU/ml inoculation solution	Volume (ml/100 gr)	Total CFU
1	<i>Bacillus coagulans BC4</i>	1:1,000	Long	6.70E+07	5	3.35E+08
2	<i>Bacillus coagulans BC30</i>	1:100	Long	4.58E+07	5	2.29E+08
3	<i>Bacillus coagulans BC01</i>	1:2,000	Long	8.80E+07	5	4.40E+08
4	<i>Bacillus subtilis Bss-19</i>	1:2,000	Long	5.97E+07	5	2.98E+08
5	<i>Bacillus subtilis HU58</i>	1:1,000	Long	6.78E+07	5	3.39E+08
6	<i>Bacillus subtilis DE111</i>	1:1,000	Long	7.00E+07	5	3.50E+08
7	<i>Bacillus subtilis MB40</i>	1:1,000	Long	6.80E+07	5	3.40E+08
8	<i>Lactobacillus plantarum</i>	1:10,000	Long	3.72E+07	5	1.86E+08
9	<i>Lactobacillus paracasei</i>	1:1,000	Long	3.68E+08	5	1.84E+09
10	<i>L. rhamnosus HN001</i>	1:10,000	Long	6.57E+07	5	3.28E+08
11	<i>Lactobacillus acidophilus</i>	1:2,000	Long	9.50E+07	5	4.75E+08
12	N.C	-	-	-	-	-

Table 6. Results in inoculated mushrooms

24 h		Middle (4-6 days)				End of shelf-life (11 days)			
Probiotic enrichment		Probiotic enrichment				Probiotic enrichment			
Efficiency	Enriched reps	Total P-CFU/gr	Efficiency	Enriched reps	Total P-CFU/gr	Efficiency	Enriched reps	Total P-CFU/gr	Enriched reps
57.38% A	4/4	2.75E+06 A	81.94% AB	4/4	**3.15E+06 A	137.26% A	4/4	**3.15E+06 A	4/4
55.01% A	4/4	1.06E+06 CD	46.05% B	4/4	1.62E+06 AB	70.59% BC	4/4	1.62E+06 AB	4/4
25.42% CD	4/4	9.56E+05 CDE	21.73%	4/4	1.43E+06 AB	32.43% CDE	4/4	1.43E+06 AB	4/4
30.84% BC	4/4	**2.24E+06 AB	75.03% A	4/4	**2.30E+06 AB	77.14% B	4/4	**2.30E+06 AB	4/4
40.50% B	4/4	*8.85E+05 CDEF	26.10% BC	4/4	**1.81E+06 AB	53.27% BCD	4/4	**1.81E+06 AB	4/4
30.79% BC	4/4	*1.60E+06 BC	45.609% B	4/4	**2.35E+06 AB	67.13% BC	4/4	**2.35E+06 AB	4/4
17.79% DE	4/4	1.40E+05 EF	4.11% C	4/4	6.23E+05 AB	18.32% DE	4/4	6.23E+05 AB	4/4
15.59% DEF	4/4	8.58E+04 F	4.62% C	4/4	2.77E+05 C	14.92% DE	4/4	2.77E+05 C	4/4
1.66% G	4/4	**2.41E+05 DEF	1.31% C	4/4	**3.13E+06 A	16.99% DE	4/4	**3.13E+06 A	4/4
5.26% EFG	4/4	*4.08E+04 EF	1.24% C	4/4	**8.28E+05 AB	25.21% DE	4/4	**8.28E+05 AB	4/4
3.28% FG	4/4	1.29E+05 EF	2.71% C	4/4	2.13E+05 C	4.49% E	4/4	2.13E+05 C	4/4
-	0/4	0.00E+00	-	0/4	0.00E+00	-	0/4	0.00E+00	0/4

Protocol							
Probiotic	Dilution	Disinfection	CFU/ml inoculation solution	Volume (ml/100 gr)	Total CFU	Total P-CFU/gr	
<i>Bacillus coagulans BC4</i>	1:1,000	Long	6.70E+07	5	3.35E+08	*1.92E+06 A	
<i>Bacillus coagulans BC30</i>	1:100	Long	4.58E+07	5	2.29E+08	1.26E+06 BC	
<i>Bacillus coagulans BC01</i>	1:2,000	Long	8.80E+07	5	4.40E+08	1.12E+06 BC	
<i>Bacillus subtilis Bss-19</i>	1:2,000	Long	5.97E+07	5	2.98E+08	*9.20E+05 CD	
<i>Bacillus subtilis HU58</i>	1:1,000	Long	6.78E+07	5	3.39E+08	1.37E+06 B	
<i>Bacillus subtilis DE111</i>	1:1,000	Long	7.00E+07	5	3.50E+08	*1.08E+06 BC	
<i>Bacillus subtilis MB40</i>	1:1,000	Long	6.80E+07	5	3.40E+08	6.05E+05 DE	
<i>Lactobacillus plantarum</i>	1:10,000	Long	3.72E+07	5	1.86E+08	2.90E+05 EF	
<i>Lactobacillus paracasei</i>	1:1,000	Long	3.68E+08	5	1.84E+09	*3.06E+05 EF	
<i>L. rhamnosus HN001</i>	1:10,000	Long	6.57E+07	5	3.28E+08	*1.73E+05 F	
<i>Lactobacillus acidophilus</i>	1:2,000	Long	9.50E+07	5	4.75E+08	1.56E+05 F	
N.C	-	-	-	-	-	0.00E+00	

	Treatment	1	2	3	4	5	6	7	8	9	10	11	12

[0149] To summarize, the inventors showed that in the case of lettuce and carrots, vegetative probiotic bacterial strains did not penetrate the plant tissue (did not survive surface disinfection).

[0150] There were no principal differences between subterminal and free endospores. In the current study, *Bacillus coagulans* BC01 and *Bacillus subtilis* MB40 had somewhat lower results.

[0151] In lettuce, the CFU/gr did not change over storage time. In carrots, the inventors observed a significant growth in CFU/gr for almost all strains.

[0152] In the case of mushrooms, in contrast to lettuce and/or carrots, the inventors showed that vegetative probiotic bacterial strains penetrated the tissue and survived the storage time. In general, endospore-forming strains were found to penetrate mushrooms with increased penetration numbers and/or efficiency. Six out of the eleven tested probiotic bacterial strains (endospores and vegetative) increased their CFU/gr over the storage time.

[0153] Although the length of the various vegetative cells tested (**Table 3**) was significantly smaller than that of BC4 endospores, surprisingly their penetration into plant tissue was significantly lower, demonstrating. This result is clearly unexpected.

EXAMPLE 4

[0154] Further to the above, the inventors have tested penetration and survival of mixtures comprising different combinations of probiotic bacterial strains in vegetative and spore forms. Specifically mixes including 0%, 25%, 50%, 75%, or 100% *B. coagulans* BC4 in endospore form (complemented to 100% with vegetative *B. coagulans* BC4) were prepared. Penetration and survival of the different preparations were tested in lettuce, carrots, and mushrooms, in time 0, after 24 hr, 48 hr, 'middle' (after 6 days) and 'end of shelf life' (after 10 days).

[0155] In lettuce, the inventors showed optimal penetration is achieved for ~75% endospores (in-line with previous results disclosed herein). Regarding the survivability, the inventors showed that when endospores are present in an amount of 25% or higher, there is no change over time in endophytic survivability (**Fig. 3B**). The threshold for endophytic survivability is 25% endospores (**Fig. 3B**) and for total product survivability is 50% endospores (**Fig. 3A**). Further, the inventors

showed that 100% of vegetative *BC4* did not survive for more than 48 hours as endophytes (**Fig. 3B**) but did survive as epiphytes until day 6 (**Fig. 3A**).

[0156] In carrots, the inventors showed optimal penetration is achieved for ~100% endospores (though, no significant differences were observed between 75% and 100%). Regarding the survival, the inventors showed a general increase in PCFU/gr over the 14 days of storage (**Fig. 4A**). The endophytic survival was found to be significantly affected by the endospores' %. Specifically, 100% endospores solution provided the best PCFU /gr results over storage time (**Fig. 4B**). For total product CFU/gr, % between 50% and 75% of endospores in the inoculation solution seems to be a threshold required to achieve improved or superior total product CFU/gr (**Fig. 4B**). When endospores are present in an amount of 50% or more, an increase in both total and endophytic PCFU/gr was observed over time (**Figs. 4A-4B**, respectively).

[0157] In mushrooms, the inventors showed that the optimal penetration is achieved for ~75% endospores (though, no significant differences were observed between 25% and 100%). Regarding survivability, the inventors showed that for 100% vegetative *B4* inoculation solution, no surviving *BC4* cells are observed after 7 days (**Fig. 5A**). No change in endophytic PCFU/gr was observed over time for endospores % being 25% or higher in the inoculation solution (excluding 100% at $t=0$; **Fig. 5B**). Inoculation solutions of 25%, 50%, 75% and 100% *BC4* endospores showed a significant decrease only in total CFU/gr over time (**Fig. 5A**), but not in the endophytic CFU/gr (**Fig. 5B**).

EXAMPLE 5

[0158] The inventors further compared subterminal endospores to free endospores.

[0159] CFU/ml of inoculation solution among the different treatments was essentially the same.

[0160] In lettuce, the inventors showed that penetration for the subterminal endospore was rather high (**Table 7** and **Fig. 6A**). Free spores showed a significant increase of *BC4* CFU/gr (endophytic) after 12 days of storage (**Table 7** and **Fig. 6A**).

[0161] In carrots, the inventors showed that the subterminal endospores inoculation solution presented high penetration efficiency, and endophytic survivability in the early stages of the storage (**Table 8** and **Fig. 6B**). All treatments showed a significant increase of *BC4* CFU/gr after 15 days of storage. Of notion is the total CFU/gr of the subterminal endospores treatment at the end of shelf life (**Table 8** and **Fig. 6B**).

Table 7. Results in lettuce inoculated with subterminal spores vs. free spores

Total P-CFU/gr									
T= 0		T= 1 day			T= middle (6 days)			T= end (12 days)	
Probiotic enrichment		Probiotic enrichment			Probiotic enrichment			Probiotic enrichment	
Efficiency	Enriched step	Total P-CFU/gr	Efficiency	Enriched step	Total P-CFU/gr	Efficiency	Enriched step	Total P-CFU/gr	Efficiency
41.7%	4/4	1.61E+06	58.5%	4/4	1.52E+06	55.1%	4/4	1.78E+06	64.6%
37.1%	4/4	1.54E+06	57.2%	4/4	1.11E+06	41.0%	4/4	1.09E+06	40.5%
2.9%	4/4	3.29E+05	9.3%	4/4	2.55E+05	7.2%	4/4	1.83E+05	5.2% B
0.8%	4/4	2.47E+05	6.7%	4/4	1.77E+05	4.8%	4/4	3.12E+05	8.4% A
-	0/4	0.00E+00	-	0/4	0.00E+00	-	0/4	0.00E+00	-
									0/4

Protocol								
Combination	Dilution	Endospore forming	CFU/ml inoculation solution	Disinfection	Volume (ml/100 gr)	Total CFU/ml	Inoculation time (min)	Total P- CFU/gr
L.1 × BC4	1:1000	subterminal	7.32E+07	No disinfection	5	3.66E+08	30	1.15E+06
L.1 × BC4	1:1000	free	7.10E+07	No disinfection	5	3.55E+08	30	1.00E+06
L.1 × BC4	1:1000	subterminal	7.32E+07	Long	5	3.66E+08	30	1.03E+05
L.1 × BC4	1:1000	free	7.10E+07	Long	5	3.55E+08	30	3.05E+04
L.1 × N.C	-	-	-	No disinfection	-	-	30	0.00E+00

	Treatment					
		1	2	3	4	5

Table 8. Results in baby carrots inoculated with subterminal spores vs. free spores

Total P-CFU/gr									
T= 1 day			T= middle (6 days)			T= end (12 days)			
Probiotic enrichment			Probiotic enrichment			Probiotic enrichment			
Efficiency	Enriched step	Total P-CFU/gr	Efficiency	Enriched step	Total P-CFU/gr	Efficiency	Enriched step	Total P-CFU/gr	Enriched step
39.2%	4/4	1.50E+06	54.5%	4/4	**4.71E+06 A	171.3% A	4/4		4/4
33.5%	4/4	1.45E+06	53.8%	4/4		79.4% B	4/4		4/4
16.2% A	4/4	7.89E+05	22.3%	4/4		27.1%	4/4		4/4
5.9% B	4/4	6.79E+05	18.3%	4/4		22.2%	4/4		4/4
-	0/4	0.00E+00	-	0/4		-	0/4	0.00E+00	0/4

Protocol						T = 0			
						Probiotic enrichment			
Endospore forming	CFU/ml inoculation solution	Disinfection	Volume (ml/100 gr)	Total CFU/ml	Inoculation time (min)	Total P- CFU/gr	Efficiency	Enriched step	Total P- CFU/gr
subterminal	7.32E+07	No disinfection	5	3.66E+08	30	5.13E+05	18.7%	4/4	1.08E+06
free	7.10E+07	No disinfection	5	3.55E+08	30	4.84E+05	17.9%	4/4	9.05E+05
subterminal	7.32E+07	Long	5	3.66E+08	30	3.50E+05	9.9% A	4/4	5.74E+05
free	7.10E+07	Long	5	3.55E+08	30	2.57E+05	6.9% B	4/4	2.20E+05
-	-	No disinfection	-	-	30	0.00E+00	-	0/4	0.00E+00

	Treatment		Combination	Dilution
	1		B. carrots × BC4	1:1000
	2		B. carrots × BC4	1:1000
	3		B. carrots × BC4	1:1000
	4		B. carrots × BC4	1:1000
	5		B. carrots × N.C	-

[0162] While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

CLAIMS

What is claimed is:

1. A fresh produce comprising a *Bacillus* strain colonizing a tissue of said fresh produce, wherein said *Bacillus* bacterial strain is *Bacillus coagulans*.
2. The fresh produce of claim 1, wherein at least 20% of colony forming unit (CFU) of said *Bacillus* strain colonizing said tissue of said fresh produce are in a spore form.
3. The fresh produce of claim 1 or 2, wherein said *Bacillus* strain is a *B. coagulans* strain.
4. A fresh produce comprising a spore-forming probiotic bacterial strain, colonizing a tissue of said fresh produce, wherein at least 20% of CFU of said spore-forming bacterial strain colonizing said tissue of said fresh produce are in a spore form.
5. The fresh produce of claim 4, wherein said probiotic bacterial strain is selected from the group consisting of: *B. coagulans*, *B. subtilis*, *B. indicus*, *B. licheniformis*, *B. clausii*, and any combination thereof.
6. The fresh produce of claim 5, wherein said *Bacillus* strain is a *B. coagulans* strain.
7. The fresh produce of any one of claims 1 to 6, wherein said strain is *B. coagulans* strain being selected from: BC4, BC30, or a combination thereof.
8. The fresh produce of any one of claims 1 to 7, comprising at least 1×10^5 CFU of said strain per 1 gr by weight of said fresh produce.
9. The fresh produce of any one of claims 1 to 8, wherein said strain colonizes an intercellular space, an intracellular space, or both, of said tissue.
10. The fresh produce of any one of any one of claims 1 to 9, wherein said strain constitutes at least 10% of bacteria colonizing said tissue of said fresh produce.
11. The fresh produce of any one of claims 1 to 10, wherein said strain is probiotic to a subject consuming thereof.
12. The fresh produce of any one of claims 1 to 11, wherein said strain is an exogenous endophyte of said fresh produce.

13. The fresh produce of claim 12, wherein at least 5% of CFU of said strain survive surface disinfection of said fresh produce, thereby determined as an exogenous endophyte of said fresh produce.

14. The fresh produce of any one of claims 1 to 13, being a processed product.

15. The fresh produce of claim 14, wherein said processed product is cut, sterilized, washed, or any combination thereof.

16. The fresh produce of claim 15, wherein said sterilized product underwent surface sterilization.

17. The fresh produce of any one of claims 1 to 16, wherein said strain is exogenously applied to said fresh produce pre-harvest, post-harvest, or both.

18. The fresh produce of any one of claims 1 to 17, wherein said fresh produce is selected from the group consisting of: a plant or plant material, a fungus or fungus material, and any combination thereof.

19. The fresh produce of claim 18, wherein said plant or plant material comprises any one of: leaf, fruit, root, tuber, bulb, stem, seed, and any combination thereof.

20. The fresh produce of any one of claims 1 to 19, being a leafy green.

21. The fresh produce of any one of claims 1 to 20, for use in prevention or treatment of dysbiosis in a subject in need thereof.

22. A kit comprising:

a. a fresh produce comprising a spore-forming bacterial strain, colonizing a tissue of said fresh produce, wherein at least 20% of CFU of said spore-forming bacterial strain colonizing said tissue of said fresh produce are in a spore form; and

b. a container configured to store said fresh produce of (a) under conditions such that at least 20% of CFU of said spore-forming bacterial strain colonizing said tissue of said fresh produce are maintained in a spore form.

23. The kit of claim 22, wherein said spore-forming bacterial strain is capable of improving health of a subject consuming thereof.

24. The kit of claim 22 or 23, wherein said container is further configured to store said fresh produce under conditions such that said fresh produce comprising said spore-forming bacterial strain is capable of improving health of a subject consuming thereof.

25. A method for preparing the fresh produce of any one of claims 1 to 19, the method comprising contacting a fresh produce with an effective amount of a composition comprising a spore-forming bacterial strain, thereby preparing the fresh produce.

26. The method of claim 25, wherein at least 20% of CFU of said spore-forming bacterial strain of said composition are in a spore form.

27. The method of claim 25 or 26, wherein said composition comprises at least one agent capable of maintaining at least 20% of CFU of said spore-forming bacterial strain of said composition in a spore form.

28. The method of claim 27, wherein said agent is selected from the group consisting of: surfactant, pH adjuster, and a combination thereof.

29. The method of any one of claims 25 to 28, wherein said contacting is pre-harvest contacting, post-harvest contacting, or both.

30. The method of any one of claims 25 to 29, wherein said contacting is post-harvest contacting, and said method further comprises a step before said contacting comprising disinfecting or sterilizing said fresh produce.

31. The method of claim 30, wherein said sterilizing comprises sterilizing a surface of said fresh produce.

32. The method of any one of claims 25 to 31, wherein said contacting is spraying.

33. The method of any one of claims 25 to 32, wherein said composition is formulated for spraying.

34. The method of any one of claims 25 to 33, wherein said fresh produce is selected from the group consisting of: a plant or plant material, a fungus or fungus material, and any combination thereof.

35. The method of claim 34, wherein said plant or plant material comprises any one of: leaf, fruit, root, tuber, bulb, stem, seed, and any combination thereof.

36. The fresh produce of any one of claims 25 to 35, being a leafy green.