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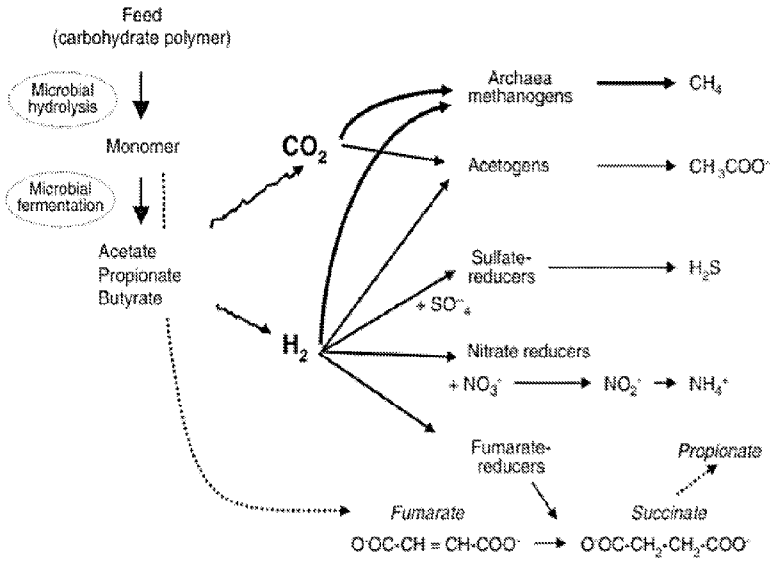

FIG. 1

(57) Abstract: The subject invention provides compositions and methods for feeding livestock animals and reducing greenhouse gas emissions from livestock animals. The compositions comprise one or more beneficial microorganisms and/or growth by-products thereof, as well as one or more nutritive mineral components. In certain embodiments, the composition is formulated as a compressed mineral assembly, such as a salt lick or mineral lick, which, when made available to a livestock animal, can be ingested by the livestock animal.

WO 2022/006121 A1

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IMPROVED FEED BLOCK SUPPLEMENTS FOR LIVESTOCK HEALTH AND METHANE REDUCTION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application No. 63/046,320, filed June 30, 2020, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

In the breeding and raising of cattle and other livestock, a certain amount of salt is necessary to be absorbed with other sources of nutrients. Salt is the primary source of the essential nutrients sodium and chloride. As electrolytes in the body, sodium and chloride play many important biological roles in maintaining fluid and acid-base balance through kidney function, facilitating nutrient absorption and promoting cellular function through establishment of membrane electrochemical gradients.

Animals have a daily requirement for sodium and chloride, which can be met by consuming salt. The recommended salt intake is between 0.25 and 0.5% of the total consumed diet dry matter. Forages contain very little sodium (<0.01%), so salt is often added to a concentrate or offered "free choice," meaning made available for the animals to use at their own will.

Animals have an appetite for salt and will seek out sources of salt if not adequately supplied in their diet. One sign of salt deficiency is pica, a term for abnormal eating behavior. Salt deficient animals may consume dirt, drink urine and chew on rocks, pipes and wood in an effort to meet their craving for salt. Pica is not exclusive to salt deficiency, however. Iron, phosphorus and potassium deficiencies can all induce pica as well. Other signs of salt deficiency can include weight loss, appetite loss, depleted appearance, reduced milk production, and reduced performance.

Salt supplements come in two forms, loose granular and compressed block products (i.e., salt licks, lick blocks, salt blocks, and mineral licks). Free choice salt feeding is the easiest method to provide salt, especially out on a pasture. To be consumed, salt blocks need to be licked. In contrast, loose granular salt can be licked or chewed (bites of salt). The type of salt complex utilized can depend upon many factors, including the species of animal. For example, llamas and alpacas do not lick like sheep, horses and cattle, and thus require granule (chewable) forms of salt to meet their nutritional needs.

Besides the physical form of salt supplements, they can fundamentally vary in composition, which is identified by different colors. Free choice salt products typically are either white or red in color. White salt contains pure sodium chloride and no other mineral

sources. Red salt is typically a trace mineral salt product meaning that other minerals, primarily trace minerals like copper, cobalt, iodine, iron, selenium and zinc, are added and salt is acting as a carrier. Ferric oxide is added to impart the red color to trace mineral salt. Unlike that for sodium, animals do not have specific appetites for trace minerals and sodium is used to facilitate intake.

Another form of feed supplement, similar to a salt block, is a high-quality feed block (HQFB). These solid blocks can help improve digestion, lactation, reproduction and weight gain. The general formula of HQFB includes molasses, non-protein nitrogen, rumen by-pass protein, minerals, vitamins and lipids, although other binders, preservatives, and energy-rich components can also be utilized. For example, as an alternative to molasses, the main ingredient may vary from date pulp, to rice bran poultry waste, tomato pulp, olive cake, to brewery grains.

Proper nutrition is critical for maintaining healthy and productive livestock animals. Another area in which livestock nutrition has gained importance is in environmental protection, notably, greenhouse gas (GHG) reduction. Methane, carbon dioxide and nitrous oxide are all produced as a result of livestock production.

Ruminants such as, for example, cattle, sheep, buffalo, goats, deer and camels, are unique because of their four stomach compartments: the reticulum, rumen, omasum and abomasum. The rumen, in particular, is a large, hollow organ where microbial fermentation of ingested substances, such as fibrous plant material, occurs. This organ can hold 40-60 gallons of material, with an estimated microbial concentration of 150 billion microbes per teaspoon of rumen contents.

The rumen functions as an anaerobic fermentation vessel for certain bacteria that produce gaseous fermentation by-products, including oxygen, nitrogen, H_2 and carbon dioxide. *See* **FIG. 1.** Methanogenesis is a natural process contributing to the efficiency of the digestive system, reducing the partial pressure of H_2 and allowing the normal functioning of microbial enzymes. The process is regulated by methanogens, the most common of which is *Methanobrevibacter*. Methanogens form a biofilm on surfaces where hydrogen-producing bacteria and protozoa actively produce H_2 required for reducing carbon dioxide to methane.

As an example, cattle, raised for both beef and milk, as well as for inedible outputs like manure and draft power, are responsible for the greatest amounts of emissions from livestock, representing about 65% of the livestock sector's emissions. Approximately 130 to over 250 gallons of ruminal gas produced by fermentation can be belched from one cow each day. This is important for the health of the cow, as it prevents bloating; however, the negative result is the emission of GHG such as carbon dioxide and methane into the atmosphere.

Other animals, including non-ruminant animals, also contribute to enteric GHG production. For example, swine, rodents, monkeys, horses, mules, asses, rhinoceros, hippopotamuses, bears, poultry and certain other birds also contain methanogenic bacteria in their digestive systems.

In addition to gut fermentation, livestock manure can also be a source of GHG emissions. Manure contains two components that can lead to GHG emissions during storage and processing: organic matter that can be converted into methane emissions, and nitrogen that leads indirectly to nitrous oxide emissions. Methane is released when methanogenic bacteria decompose the organic material in the manure as it is being held in lagoons, tailing ponds or holding tanks. Additionally, nitrogen in the form of ammonia (NH_3) is released from manure and urine during storage and processing. Ammonia can later be transformed into nitrous oxide. (Gerber et al. 2013).

Currently, approaches for reducing livestock methane emissions include defaunation of the digestive system and even vaccination against methanogens. The downsides to these strategies, however, are that they may reduce the number of beneficial gut microbes, and the methods may be short-lived due to microbial adaptation. Additionally, energy providers have attempted to harvest methane from manure lagoons and collection ponds as a form of biogas fuel; however, the methods are inefficient and do not capture significant amounts of methane relative to the total amount of methane produced by livestock production.

Other strategies have involved dietary modification, particularly for livestock grazing pasture, in order to manipulate gut fermentation by, for example, directly inhibiting methanogens and protozoa, or by redirecting hydrogen ions away from the methanogens to reduce methanogenesis. Such dietary modifications include, for example, the addition of probiotics, acetogens, bacteriocins, ionophores (e.g., monensin and lasalocid), organic acids and/or plant extracts (e.g., tannins and/or seaweed), to feed. (Ishler 2016). Most anti-methanogenic compounds are costly, short-lived, show inconsistent results, require high concentrations, do not contain H_2 acceptors, do not affect methanogens in the form of biofilms, and comprise compounds that are easily destroyed in the gut. Additionally, for free range livestock, there is a problem with dosing because the feed is not rationed as it is in a feedlot. The animals are free to eat as they wish, so regular administration of anti-methanogenic compounds is much more difficult.

The livestock industry is important for the production of, for example, meats, textiles and dairy products; however, growing concerns over climate change and a need for reducing GHG emissions calls for improved approaches for feeding and producing livestock with reduced GHG emissions.

BRIEF SUMMARY OF THE INVENTION

The subject invention provides compositions and methods for feeding livestock and other animals. More specifically, the subject invention provides compositions that, when contacted with the digestive system and/or waste of an animal, lead to enhanced health as well as a reduction in greenhouse gas emissions that would have otherwise been produced by the animal's digestive processes and/or waste.

In certain embodiments, the subject invention provides a digestive health composition for livestock animals, wherein the composition comprises one or more beneficial microorganisms and/or

one or more microbial growth by-products. In preferred embodiments, the composition further comprises one or more nutritive and/or mineral components, including, for example, molasses, urea, and/or sources of sodium, chloride, calcium, phosphorus, magnesium, potassium, sulfur, cobalt, copper, iron, selenium, iodine, manganese, zinc and/or other essential and/or supplemental nutrients and/or minerals.

In certain embodiments, the composition is formulated for oral administration to the animal. In preferred embodiments, the composition is formulated as a free choice nutrient and/or mineral supplement, for example, a mineral block, salt block, salt lick, high-quality feed block (HQFB), molasses block, urea-molasses-mineral block (UMMB) and/or as salt and/or mineral granules, crystals or pellets. In some embodiments, the composition is formulated as a mineral and/or salt concentrate that is mixed into an animal's feed and/or drinking water.

In certain embodiments, the beneficial microorganisms are non-pathogenic bacteria of the genus *Bacillus*. In further preferred embodiments, the composition utilizes *Bacillus* spp. that are capable of producing one or more of the following: surface active agents, such as lipopeptides and/or glycolipids; bioactive compounds with antimicrobial and/or immune-modulating effects; polyketides; acids; peptides; anti-inflammatory compounds; enzymes, such as proteases, amylases, and/or lipases; and sources of amino acids, vitamins, and other nutrients.

The bacteria can be used in spore form, as vegetative cells, and/or as a mixture thereof. Preferably, the bacteria can survive high salt, high heat and/or high pressure environments.

In a preferred embodiment, the composition comprises a strain of *B. amyloliquefaciens*. In a specific preferred embodiment, the strain of *B. amyloliquefaciens* is *B. amyloliquefaciens* "*B. amy*" (NRRL B-67928) *B. amy* is particularly advantageous over traditional probiotic microorganisms due to its ability to produce spores that remain viable in the digestive tract and, in some embodiments, after being excreted in the animal's waste. Additionally, *B. amy* is capable of surviving under high salt, high heat and high pressure, such as the levels utilized in producing salt/mineral blocks and granules. Furthermore, *B. amy* produces a unique mixture of metabolites that provide a broad-spectrum of digestive and environmental benefits when administered to a livestock animal and/or its waste. Even further, in some embodiments, *B. amy* is a nitrogen-fixer.

In certain embodiments, the composition comprises a strain of *Bacillus subtilis*. In preferred embodiments, the strain is *B. subtilis* B4 (NRRL B-68031). Advantageously, in some embodiments, B4 produces more lipopeptides biosurfactants compared to reference strains of *Bacillus subtilis*, in particular, the lipopeptide surfactin. In a specific exemplary embodiment, the composition comprises both *B. amy* and B4.

Advantageously, in addition to providing a livestock animal with necessary salt and trace minerals, the subject compositions can, in preferred embodiments, help reduce deleterious atmospheric gas emissions resulting from livestock production by controlling and/or inhibiting

methanogenic microbes, and/or symbionts thereof, present in the animal's digestive system and/or waste.

In one embodiment the composition disrupts methanogen biofilms. In one embodiment, the composition directly inhibits methanogens and/or the biological pathways involved in methanogenesis.

Advantageously, in preferred embodiments, the subject compositions can also decrease the amount of excess H_2 that may be produced when methanogenesis is inhibited, by, for example, introducing H_2 acceptors.

In one embodiment, the digestive health composition comprises a microbial growth by-product. The microbial growth by-product can be produced by the microorganisms of the composition, and/or they can be produced separately and added to the composition.

In one embodiment, the growth by-product has been purified from a cultivation medium in which it was produced. Alternatively, in one embodiment, the growth by-product is utilized in crude form. The crude form can comprise, for example, a liquid supernatant resulting from cultivation of a microbe that produces the growth by-product of interest, including residual cells and/or nutrients.

The growth by-products can include metabolites and/or other biochemicals produced as a result of cell growth, including, for example, biosurfactants, enzymes, polyketides, acids, alcohols, solvents, proteins and/or peptides.

In certain embodiments, the composition comprises a germination enhancer for enhancing germination of spore-form microorganisms upon entering the digestive system of the livestock animal. In specific embodiments, the germination enhancers are amino acids, such as, for example, L-alanine and/or L-leucine. In one embodiment, the germination enhancer is manganese.

In one embodiment, the composition comprises one or more fatty acids and/or one or more additional components known to reduce methane in the animal's digestive system. In one embodiment, the subject composition can comprise one or more additional substances and/or nutrients to supplement the animal's nutritional needs and promote health and/or well-being in the animal.

In some embodiments, the microorganisms of the composition can produce and/or provide the fatty acids, methane reducers and/or health-promoting substances and/or nutrients.

In preferred embodiments, the subject invention provides methods of feeding a livestock animal, wherein a digestive health composition according to the subject invention is made available to the animal such that the animal can ingest the composition.

In preferred embodiments, the composition is made available in the form of a mineral block, salt lick, lick block, multi-nutrient block, molasses block, UMMB, HQFB, granules, crystals or pellets, which, when placed in a location where the animal grazes, feeds or traverses, can be licked or chewed by the animal upon the animal's choosing.

As is known in the agricultural arts, the composition is preferably made available in proximity (e.g., less than 1,000 feet) to a source of drinking water.

In some embodiments, the composition is made available in the form of granules, pellets or a concentrate that are mixed with the animal's feed and/or drinking water at a pre-determined dosage.

In certain embodiments, the methods enhance the animal's health by supplementing its diet with a nutrient, a salt or another trace mineral. Thus, in some embodiments, the methods can be used to treat and/or prevent nutrient, salt and/or or other mineral deficiencies, as well as conditions caused by such deficiencies (e.g., weight loss, pica, and/or decreased milk production).

In certain embodiments, the methods enhance the animal's health by, for example, contributing to a healthy gut microbiome; improving digestion; increasing feed-to-muscle conversion ratio; increasing milk production and quality; modulating the immune system; and/or increasing life expectancy.

In certain embodiments, the methods reduce methane, carbon dioxide and/or other deleterious atmospheric gases, and/or precursors thereof (e.g., nitrogen and/or ammonia, which are precursors of nitrous oxide) that are typically produced in the digestive system and/or waste of livestock animals.

Advantageously, in preferred embodiments, the methods can result in a direct inhibition of methanogenic bacteria and/or symbionts thereof, disruption of methanogenic biofilms, and/or disruption of the biological pathway involved in methanogenesis in the animal's digestion system, for example, the rumen, stomach and/or intestines.

In certain embodiments, the methods can also counteract H_2-acceptor depletion that results from reduced methanogenesis. Accordingly, potential negative effects of excessive H_2 on livestock products can be prevented and/or reduced. For example, excess H_2 in the digestive tract of mammals can produce a fishy smell in milk due to the overproduction of trimethylamine.

In some embodiments, the methods result in increased conversion of nitrogen to muscle mass, thereby reducing the amount of nitrogen that is available for production of ammonia and nitrous oxide.

In certain embodiments, the methods can reduce methane, carbon dioxide and/or nitrous oxide emissions from the livestock's digestive processes. In certain embodiments, the methods also reduce GHG emissions from the livestock animal's waste (e.g., urine and/or manure).

In some embodiments, the beneficial microorganisms of the composition can survive transport through the digestive system and are excreted with the animal's waste, where they continue inhibiting methanogens and/or symbionts thereof, disrupting methanogenic biofilms, disrupting the biological pathways involved in methanogenesis, compensating for H_2 acceptor loss, and/or fixing nitrogen.

In some embodiments, the methods of the subject invention can be utilized by a livestock producer for reducing carbon credit usage. Thus, in certain embodiments, the subject methods can further comprise conducting measurements to assess the effect of the method on reducing the generation of methane, carbon dioxide and/or other deleterious atmospheric gases, and/or precursors thereof (e.g., nitrogen and/or ammonia), and/or to assess the effect of the method on the control of

methanogens and/or protozoa in the livestock animal's digestive system and/or waste, using standard techniques in the art.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows biological pathways involved in methanogenesis.

Figure 2 shows the results of in-vitro studies of compositions according to embodiments of the subject invention to determine their ability to reduce enteric methane emissions from cattle rumen.

Figure 3 shows the results of in-vitro studies of compositions according to embodiments of the subject invention to determine their ability to reduce enteric carbon dioxide emissions from cattle rumen.

Figure 4 shows the results of in-vitro studies of *B. amy* at variable inclusion rates to determine its ability to reduce enteric methane emissions from cattle rumen.

Figure 5 shows the results of in-vitro studies of *B. amy* at variable inclusion rates to determine its ability to reduce enteric carbon dioxide emissions from cattle rumen.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides compositions and methods for feeding animals. More specifically, the subject invention provides compositions that, when contacted with the digestive system and/or waste of a livestock animal, lead to enhanced health and nutrition, in addition to a reduction in greenhouse gas emissions that would have otherwise been produced by the animal's digestive processes.

Selected Definitions

As used herein, a "biofilm" is a complex aggregate of microorganisms, such as bacteria, wherein the cells adhere to each other and/or to a surface using, for example, an exopolysaccharide matrix. The cells in biofilms are physiologically distinct from planktonic cells of the same organism, which are single cells that can float or swim in liquid medium.

As used herein, the term "control" used in reference to an undesirable microorganism (e.g., a methanogen) extends to the act of killing, disabling, immobilizing and/or reducing the population numbers of the microorganism, and/or otherwise rendering the microorganism incapable of reproducing and/or carrying out the processes that are undesirable (e.g., methane production).

As used herein, the "digestive system" refers to the system of organs in an animal's body that enables digestion, or the consumption of food and conversion thereof to energy and waste. The digestive system can comprise, for example, an oral cavity, esophagus, crop, gizzard, proventriculus, stomach, rumen, reticulum, omasum, abomasum, pancreas, liver, small intestine, large intestine (colon), cecum, appendix, and/or anus. Additional organs or parts related to digestion and that are specific to a particular animal are also envisioned.

As used herein, "enhanced" means improved and/or increased.

As used herein, an "isolated" or "purified" nucleic acid molecule, polynucleotide, polypeptide, protein, organic compound such as a small molecule (e.g., those described below), or other compound is substantially free of other compounds, such as cellular material, with which it is associated in nature. For example, a purified or isolated polynucleotide (ribonucleic acid (RNA) or deoxyribonucleic acid (DNA)) is free of the genes or sequences that flank it in its naturally-occurring state. A purified or isolated polypeptide is free of the amino acids or sequences that flank it in its naturally-occurring state. A purified or isolated microbial strain is removed from the environment in which it exists in nature. Thus, the isolated strain may exist as, for example, a biologically pure culture, or as spores (or other forms of the strain) in association with a carrier.

In certain embodiments, purified compounds are at least 60% by weight the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. For example, a purified compound is one that is at least 90%, 91%, 92%, 93%, 94%, 95%, 98%, 99%, or 100% (w/w) of the desired compound by weight. Purity is measured by any appropriate standard method, for example, by column chromatography, thin layer chromatography, or high-performance liquid chromatography (HPLC) analysis.

As used herein, "ionophores" are carboxylic polyether non-therapeutic antibiotics that disrupt the ion concentration gradient (Ca2+, K+, H+, Na+) across microorganisms, which causes them to enter a futile ion cycle. The disruption of the ion concentration prevents the microorganism from maintaining normal metabolism and causes the microorganism to expend extra energy. Ionophores function by selecting against or negatively affecting the metabolism of gram-positive bacteria, such as methanogens, and protozoa.

A "metabolite" refers to any substance produced by metabolism (e.g., a growth by-product) or a substance necessary for taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material, an intermediate in, or an end product of metabolism. Examples of metabolites can include, but are not limited to, enzymes, toxins, acids, solvents, alcohols, proteins, carbohydrates, vitamins, minerals, microelements, amino acids, polymers, polyketides, and surfactants.

As used herein, a "methanogen" is a microorganism that produces methane gas as a by-product of metabolism. Methanogens are archaea that can be found in the digestive systems and metabolic waste of ruminant animals and non-ruminant animals (e.g., pigs, poultry and horses). Examples of methanogens include, but are not limited to, *Methanobacterium* spp. (e.g., *M. formicicum*), *Methanobrevibacter* spp. (e.g., *M. ruminantium*), *Methanococcus* spp. (e.g., *M. paripaludis*), *Methanoculleus* spp. (e.g., *M. bourgensis*), *Methanoforens* spp. (e.g., *M. stordalenmirensis*), *Methanofollis liminatans*, *Methanogenium wolfei*, *Methanomicrobium* spp. (e.g., *M. mobile*), *Methanopyrus kandleri*, *Methanoregula boonei*, *Methanosaeta* spp. (e.g., *M. concilii*, *M.*

thermophile), *Methanosarcina* spp. (e.g., *M. barkeri*, *M. mazeii*), *Methanosphaera stadtmanae*, *Methanospirillium hungatei*, *Methanothermobacter* spp., and/or *Methanothrix sochngenii*.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 as well as all intervening decimal values between the aforementioned integers such as, for example, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, and 1.9. With respect to sub-ranges, "nested sub-ranges" that extend from either end point of the range are specifically contemplated. For example, a nested sub-range of an exemplary range of 1 to 50 may comprise 1 to 10, 1 to 20, 1 to 30, and 1 to 40 in one direction, or 50 to 40, 50 to 30, 50 to 20, and 50 to 10 in the other direction.

As used herein, "reduction" means a negative alteration and "increase" means a positive alteration, wherein the positive or negative alteration is at least 0.25%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100%.

The transitional term "comprising," which is synonymous with "including," or "containing," is inclusive or open-ended and does not exclude additional, un-recited elements or method steps. By contrast, the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. Use of the term "comprising" contemplates other embodiments that "consist" or "consist essentially of" the recited component(s).

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a," "and" and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All references cited herein are hereby incorporated by reference in their entirety.

Digestive Health Compositions

In certain embodiments, the subject invention provides a digestive health composition for animals, wherein the composition comprises one or more beneficial microorganisms and/or one or

more microbial growth by-products. In preferred embodiments, the composition further comprises one or more mineral components, including, for example, molasses, urea, protein, and/or sources of sodium, chloride, calcium, phosphorus, magnesium, potassium, sulfur, cobalt, copper, iron, selenium, iodine, manganese, zinc and/or other essential and/or supplement nutrients or minerals.

In certain embodiments, the digestive health composition is a "microbe-based composition," meaning a composition that comprises components that were produced as the result of the growth of microorganisms or other cell cultures. Thus, the microbe-based composition may comprise the microbes themselves and/or by-products of microbial growth. The microbes may be in a vegetative state, in spore form, in mycelial form, in any other form of microbial propagule, or a mixture of these. The microbes may be planktonic or in a biofilm form, or a mixture of both. The by-products of growth may be, for example, metabolites, cell membrane components, expressed proteins, and/or other cellular components. The microbes may be intact or lysed. The cells may be totally absent, or present at, for example, a concentration of at least 1 x 10^3, 1 x 10^4, 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8, 1 x 10^9, 1 x 10^{10}, 1 x 10^{11}, 1 x 10^{12}, 1 x 10^{13} or more CFU per milliliter of the composition.

In certain embodiments, the composition is formulated for oral administration to the animal. In preferred embodiments, the composition is formulated as a free choice nutritional and/or mineral supplement, for example, a mineral block, salt block, salt lick, HQFB, molasses block, urea-molasses-mineral block (UMMB), multi-species block, protein block, and/or as granules, crystals or pellets. In some embodiments, the composition is formulated as a concentrate that is mixed into a livestock animals feed and/or drinking water.

Advantageously, in addition to providing an animal with supplemental and/or necessary nutrients, salts and/or trace minerals, the subject compositions can, in preferred embodiments, help reduce deleterious atmospheric gas emissions resulting from livestock production by controlling and/or inhibiting methanogenic microbes, and/or symbionts thereof, present in the animal's digestive system and/or waste.

For example, the compositions can directly inhibit or control methanogenic bacteria and/or symbionts thereof in the animal's digestive system and/or waste, as well as disrupt the integrity and/or production of biofilms formed by methanogens. Additionally, in some embodiments, the compositions can interfere with biological pathways involved in methanogenesis. Furthermore, in some embodiments, the compositions can compensate for a loss of H_2 acceptor compounds that results when methanogenesis is reduced.

In some embodiments, the composition can also enhance the growth and health of livestock, while enabling more complete transformation of protein sources in feed to reduce nitrogen release in the animals' waste in the form of, e.g., ammonia and/or urea. Advantageously, in some embodiments, this can result in reduced nitrous oxide production.

In preferred embodiments, the beneficial microorganisms of the subject compositions are non-pathogenic fungi, yeasts and/or bacteria. The beneficial microorganisms may be in an active, inactive and/or dormant. In preferred embodiments, the microorganism is one that is characterized as "generally regarded as safe," or GRAS, by the appropriate regulatory agency.

The microorganisms of the subject invention may be natural, or genetically modified microorganisms. For example, the microorganisms may be transformed with specific genes to exhibit specific characteristics. The microorganisms may also be mutants of a desired strain. As used herein, "mutant" means a strain, genetic variant or subtype of a reference microorganism, wherein the mutant has one or more genetic variations (e.g., a point mutation, missense mutation, nonsense mutation, deletion, duplication, frameshift mutation or repeat expansion) as compared to the reference microorganism. Procedures for making mutants are well known in the microbiological art. For example, UV mutagenesis and nitrosoguanidine are used extensively toward this end.

In some embodiments, the beneficial microorganisms are selected based on a natural or acquired resistance to certain antibiotics administered to a livestock animal to, for example, control pathogenic and/or deleterious microbes in the digestive system or elsewhere in the animal's body.

In some embodiments, the beneficial microorganisms of the subject composition are capable of surviving transport through the livestock animal's digestive system and are excreted in the animal's waste (e.g., manure). Thus, in certain embodiments, administering a composition according to embodiments of the subject invention to the animal can result in a reduction in GHG production in the animal's waste via inhibition of methanogens and/or symbionts thereof, disruption of methanogen biofilms, interference with biological pathways involved in methanogenesis, and compensation for H_2 acceptor loss.

In one specific embodiment, the composition comprises about 1×10^3 to about 1×10^{13}, 1×10^6 to about 1×10^{12}, about 1×10^7 to about 1×10^{11}, about 1×10^8 to about 1×10^{10}, or about 1×10^9 CFU/g of each species of microorganism present in the composition.

In certain embodiments, the amount of microorganisms in one application of the composition totals about 1 to 100 grams per head (individual animals in a herd), or about 5 to about 85 grams per head, or about 10 to about 70 grams per head.

In one embodiment, the composition comprises about 1 to 100% microorganisms total by volume, about 10 to 90%, or about 20 to 75%.

In certain preferred embodiments, the composition comprises one or more bacteria and/or growth by products thereof. Preferably, the bacteria are from the *Bacillus* genus. The bacteria can be used in spore form, as vegetative cells, and/or as a mixture thereof.

In certain embodiments, the bacteria is *Bacillus acidiceler, B. acidicola, B. acidiproducens, B. acidocaldarius, B. acidoterrestrisr, B. aeolius, B.aerius, B. aerophilus, B. agaradhaerens, B. agri, B. aidingensis, B. akibai, B. alcalophilus, B. algicola, B. alginolyticus, B. alkalidiazotrophicus, B.*

alkalinitrilicus, B. alkalisediminis, B. alkalitelluris, B. altitudinis, B. alveayuensis, B. alvei, B. amyloliquefaciens, B. a. subsp. amyloliquefaciens, B. a. subsp. plantarum, B. mylolyticus, B. andreesenii, B. aneurinilyticus, B. anthracia, B. aquimaris, B. arenosi, B. arseniciselenatis, B. arsenicus, B. aurantiacus, B. arvi, B. aryabhattai, B. asahii, B. atrophaeus, B. axarquiensis, B. azotofixans, B. azotoformans, B. badius, B. barbaricus, B. bataviensis, B. beijingensis, B. benzoevorans, B. beringensis, B. berkeleyi, B. beveridgei, B. bogoriensis, B.boroniphilus, B. borstelensis, B. brevis Migula, B. butanolivorans, B. canaveralius, B. carboniphilus, B. cecembensis, B. cellulosilyticus, B. centrosporus, B. cereus, B.chagannorensis, B. chitinolyticus, B. chondroitinus, B. choshinensis, B. chungangensis, B. cibi, B. circulans, B. clarkii, B. clausii, B. coagulans, B. coahuilensis, B. cohnii, B. composti, B. curdlanolyticus, B. cycloheptanicus, B. cytotoxicus, B. daliensis, B. decisifrondis, B. decolorationis, B. deserti, B. dipsosauri, B. drentensis, B. edaphicus, B. ehimensis, B. eiseniae, B. enclensis, B. endophyticus, B. endoradicis, B. farraginis, B. fastidiosus, B. fengqiuensis, B. firmus, B. flexus, B. foraminis, B. fordii, B. formosus, B. fortis, B. fumarioli, B. funiculus, B. fusiformis, B. galactophilus, B. galactosidilyticus, B. galliciensis, B. gelatini, B. gibsonii, B. ginsengi, B. ginsengihumi, B. ginsengisoli, B.globisporus, B. g. subsp. globisporus, B. g. subsp. marinus, B. glucanolyticus, B. gordonae, B. gottheilii, B. graminis, B. halmapalus, B. haloalkaliphilus, B. halochares, B. halodenitrificans, B. halodurans, B. halophilus, B. halosaccharovorans, B. hemicellulosilyticus, B. hemicentroti, B. herbersteinensis, B. horikoshii, B. horneckiae, B. horti, B.huizhouensis, B. humi, B. hwajinpoensis, B. idriensis, B. indicus, B. infantis, B. infernus, B. insolitus, B. invictae, B. iranensis, B. isabeliae, B. isronensis, B. jeotgali, B. kaustophilus, B. kobensis, B. kochii, B. kokeshiiformis, B. koreensis, B. korlensis, B. kribbensis, B. krulwichiae, B. laevolacticus, B. larvae, B. laterosporus, B. lautus, B. lehensis, B. lentimorbus, B. lentus, B. licheniformis, B. ligniniphilus, B. litoralis, B. locisalis, B. luciferensis, B. luteolus, B. luteus, B. macauensis, B. macerans, B. macquariensis, B. macyae, B. malacitensis, B. mannanilyticus, B. marisflavi, B. marismortui, B. marmarensis, B. massiliensis, B. megaterium, B. mesonae, B. methanolicus, B. methylotrophicus, B. migulanus, B. mojavensis, B. mucilaginosus, B. muralis, B. murimartini, B. mycoides, B. naganoensis, B. nanhaiensis, B. nanhaiisediminis, B. nealsonii, B. neidei, B. neizhouensis, B. niabensis, B. niacini, B. novalis, B. oceanisediminis, B. odysseyi, B. okhensis, B. okuhidensis, B. oleronius, B. oryzaecorticis, B. oshimensis, B. pabuli, B. pakistanensis, B. pallidus, B. pallidus, B. panacisoli, B. panaciterrae, B. pantothenticus, B. parabrevis, B. paraflexus, B. pasteurii, B. patagoniensis, B. peoriae, B. persepolensis, B. persicus, B. pervagus, B. plakortidis, B. pocheonensis, B. polygoni, B. polymyxa, B. popilliae, B. pseudalcalophilus, B. pseudofirmus, B. pseudomycoides, B. psychrodurans, B. psychrophilus, B. psychrosaccharolyticus, B. psychrotolerans, B. pulvifaciens, B. pumilus, B. purgationiresistens, B. pycnus, B. qingdaonensis, B. qingshengii, B. reuszeri, B. rhizosphaerae, B. rigui, B. ruris, B. safensis, B. salarius, B. salexigens, B. saliphilus, B. schlegelii, B. sediminis, B. selenatarsenatis, B. selenitireducens, B. seohaeanensis, B.shacheensis, B. shackletonii, B. siamensis, B. silvestris, B. simplex, B. siralis, B. smithii, B. soli, B. solimangrovi, B.

solisalsi, B. songklensis, B. sonorensis, B. sphaericus, B. sporothermodurans, B. stearothermophilus, B. stratosphericus, B. subterraneus, B. subtilis, B. s. subsp. inaquosorum, B. s. subsp. spizizenii, B. s. subsp. subtilis, B. taeanensis, B. tequilensis, B. thermantarcticus, B. thermoaerophilus, B. thermoamylovorans, B. thermocatenulatus, B. thermocloacae, B. thermocopriae, B. thermodenitrificans, B. thermoglucosidasius, B. thermolactis, B. thermoleovorans, B. thermophilus, B. thermoruber, B. thermosphaericus, B. thiaminolyticus, B. thioparans, B. thuringiensis, B. tianshenii, B. trypoxylicola, B. tusciae, B. validus, B. vallismortis, B. vedderi, B. velezensis, B. vietnamensis, B. vireti, B. vulcani, B. wakoensis, B. weihenstephanensis, B. xiamenensis, B. xiaoxiensis, and/or *B. zhanjiangensis.*

In certain embodiments, the *Bacillus* is *B. amyloliquefaciens*, *B. subtilis* and/or *B. licheniformis*.

In a specific embodiment, the composition comprises *B. amyloliquefaciens*. In a specific preferred embodiment, the strain of *B. amyloliquefaciens* is *B. amyloliquefaciens* "*B. amy*" (NRRL B-67928).

In another specific embodiment, the composition comprises *B. subtilis* B4 (NRRL B-68031).

Cultures of the *B. amy* and B4 strains have been deposited with the Agricultural Research Service Northern Regional Research Laboratory (NRRL), 1400 Independence Ave., S.W., Washington, DC, 20250, USA. The *B. amy* deposit has been assigned accession number NRRL B-67928 by the depository and was deposited on February 26, 2020. The B4 deposit has been assigned accession number NRRL B-68031 by the depository and was deposited on May 6, 2021.

Each of the subject cultures has been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C 122. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, each of the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture. The depositor acknowledges the duty to replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing it.

In one embodiment, the microbe-based composition comprises a microbial growth by-product. The microbial growth by-product can be produced by the microorganisms of the composition, and/or they can be produced separately and added to the composition.

In one embodiment, the growth by-product has been purified from the cultivation medium in which it was produced. Alternatively, in one embodiment, the growth by-product is utilized in crude form. The crude form can comprise, for example, a liquid supernatant resulting from cultivation of a microbe that produces the growth by-product of interest, including residual cells and/or nutrients.

The growth by-products can include metabolites or other biochemicals produced as a result of cell growth, including, for example, amino acids, peptides, polyketides, antibiotics, proteins, enzymes, biosurfactants, solvents, vitamins, and/or other metabolites.

The microorganism(s) and/or growth by-product(s) present in the composition can be useful for inhibiting methanogens and/or the methanogenesis pathway, disrupting methanogen biofilms, and/or reducing H_2 accumulation in a livestock animal's digestive system. Furthermore, in preferred embodiments, the composition can be useful for enhancing the overall health of a livestock animal.

Bacillus spp. bacteria

In certain embodiments, the composition comprises *B. amy* and/or growth by-products thereof. *B. amy* is particularly advantageous over traditional probiotic microorganisms due to its ability to produce spores that remain viable in the digestive tract and, in some embodiments, after excretion in the animal's waste. Additionally, *B. amy* is capable of surviving under conditions of high salt, high heat and high pressures, such as those often utilized in producing compressed salt compositions. For example, *B. amy* spores can, in some embodiments, exhibit resistance to temperatures of at least 55 °C to 100 °C, or at least 80 °C to 125 °C; pressures of at least 200 MPa to 300 MPa, or at least 250 MPa to 350 MPa; and salt concentrations of, for example, 1-15% or higher, e.g., at least 5%, 10%, 12%, 15% or more. In some instances, *B. amy* is also a nitrogen-fixer.

Furthermore, *B. amy* produces a unique mixture of metabolites that provide a broad-spectrum of digestive and environmental benefits when administered to a livestock animal and/or its waste. As exemplified in Table 1 below, the growth by-products can directly inhibit methanogens, disrupt methanogen biofilms, and/or reduce H_2 concentration in a livestock animal's digestive system.

Table 1. Exemplary *B. amy* growth by-products for reducing methanogenesis and H_2

Function(s)	Growth by-product(s)	Examples (Produced by *B. amy*)
Inhibition of methanogens	Enzymes	Proteinase K (and/or a homolog thereof): can specifically lyse pseudomurien, a major structural cell wall component of some archaea, including methanogens.

		Diglycolic acid dehydrogenase (DGADH), (and/or a homolog thereof): can disrupt ether bonds between the glycerol backbone and fatty acids of the phospholipid layer of archaeal cell membranes.
	Organic acids	Propionic acid and/or acetic acid: can disrupt the structure of archaeal cell membranes.
Disruption of methanogen biofilms	Lipopeptide biosurfactants; Glycolipid biosurfactants; Polyketides	Surfactin, fengycin, iturin, bacillomycin, lichenysin, difficidin, and/or a maltose-based glycolipid: can interfere with the production and/or maintenance of the exopolysaccharide matrix that forms biofilms, thereby interfering with formation and/or adhesion of capabilities of the biofilm.
Reduction of H_2	Organic acids	Propionic acid: can stimulate acetogenic microorganisms, which produce acetic acid from hydrogen and carbon dioxide. This results in reduced hydrogen availability for methanogenic microbes to carry out methanogenesis, and also helps keep H_2 concentrations from increasing when methanogenesis decreases. Increased H_2 can lead to a build-up of trimethylamine in the digestive system, which causes a "fishy" smell in produced milk.

In one embodiment, as exemplified in Table 2 below, the composition comprises *B. amy*, and/or growth by-products thereof, which can enhance the overall health and productivity of a livestock animal by performing a variety of health-promoting functions. Thus, in some embodiments, *B. amy* can serve as a probiotic when administered to an animal.

Table 2. Exemplary *B. amy* growth by-products for enhancing livestock health

Function(s)	Growth by-product(s)	Examples (Produced by *B. amy*)
Regulation of gut microbiome	Biosurfactants; Natural antibiotics; Organic acids	Biosurfactants, including lipopeptides and glycolipids, as well as natural antibiotics (e.g., polyketides, penicillins, cephalosporins, validamycins, carbapanems, and nocardicins): can inhibit the growth of pathogenic, or otherwise deleterious gut microorganisms (e.g., *Anaeroplasma, Acholeplasma* and certain fungi) by, for example, interfering with the pathogenic or deleterious microorganism's cell membrane and/or biofilm structure. Organic acids, such as propionic acid: can promote the growth of beneficial gut microorganisms (e.g., *Proteobacteria, Rhodospirillaceae, Campylobacterales* and *Butyricimonas*) by, for example, altering the pH of the digestive system to a

		more favorable environment for such growth. In certain embodiments, regulation of the gut microbiome also leads to a reduction in nitrous oxide emissions due to a reduction in ammonia-oxidizing gut bacteria.
Stimulation of growth hormones (e.g., GH/IGH-1); increasing the rate of weight gain; and increasing feed-to-muscle conversion through improved digestion	Organic acids; Biosurfactants; Digestive enzymes	Organic acids, such as the short-chain fatty acids butyrate and valerate: can improve digestion through, for example, improved intestinal and/or ruminal cell function. Lipopeptide and glycolipid biosurfactants: can improve digestion by, for example, enhancing the bioavailability of nutrients and water through intestinal/ruminal cells and improve absorption thereof into the bloodstream. Digestive enzymes, such as amylases, lipases, and proteases (e.g., collagenase-like protease, peptidase E (N-terminal Asp-specific dipeptidase), peptidase s8 (subtilisin-like serine peptidase), serine peptidase, and endopeptidase La): can improve conversion of feed to muscle by increasing digestion of proteins, fats and carbohydrates in feed that can otherwise be difficult or impossible for the animal to digest. Additionally, because nitrogen is required for conversion of feed to muscle mass, increased nitrogen uptake in the digestive system due to improved muscle conversion can result in fewer nitrous oxide precursors, and accordingly, fewer nitrous oxide emissions.
Improving quantity and quality of produced milk in mammals	Lignocellulytic enzymes; Folic acid/folate	Lignocellulytic enzymes, such as cellulose, xylanase, laccase, and manganese catalase: can enhance digestion of polysaccharides, such as cellulose, xylan, hemicellulose, and lignin, into the components necessary for milk production. Folate: can help increase milk production by, for example, enhancing mammary gland metabolism. Additionally, folate is an important nutrient for, e.g., growth and neural development. Thus, increased folate in produced milk can improve the nutritional quality of the milk for nursing offspring, thereby potentially shortening the time required for weaning and/or increasing the growth and survival rate of offspring.
Enhancing immune health, life expectancy and overall health	Vitamins	Riboflavin, produced via riboflavin synthase: can provide antinociception and anti-inflammatory effects in a livestock animal.

		Folate, produced via bifunctional folate synthesis protein: can help regulate energy conversion, gene expression and DNA production, in addition to being an anti-inflammatory agent. Ubiquinone (CoQ10), produced via ubiquinone biosynthesis O-methyltransferase: can, as an antioxidant, prevent low-density lipoprotein oxidation, which can result in atherosclerosis.

In some embodiments, the composition can comprise other species of *Bacillus*, such as, for example, *B. licheniformis* and/or *B. subtilis*. In some embodiments, *B. licheniformis* and/or *B. subtilis* can reduce methane production by methanogens, and inhibit the methanogenic bacteria themselves through production of propionic acid and other metabolites, such as lipopeptide biosurfactants. Additionally, *B. licheniformis* can help decrease the concentration of ammonia in cattle ruminal fluids while helping increase milk protein production. In some embodiments, *B. licheniformis* and/or *B. subtilis* can help increase fecal *Lactobacillus* counts, increase the digestibility of nitrogen, and a decrease the emission of ammonia and mercaptans.

In certain embodiments, the composition can comprise *B. subtilis* B4. Strain B4 can produce lipopeptide biosurfactants in enhanced amounts, particularly surfactin. Advantageously, in some embodiments, B4 and/or the enhanced amounts of surfactin that it produces, can be especially helpful for enhanced disruption of methanogenic biofilms in livestock digestive tracts.

In some embodiments, B4 is "surfactant over-producing." For example, the strain may produce at least 0.1-10 g/L, e.g., 0.5-1 g/L biosurfactant, or, e.g., at least 10%, 25%, 50%, 100%, 2-fold, 5-fold, 7.5 fold, 10-fold, 12-fold, 15-fold or more compared to other *B. subtilis* bacteria. For example, in some embodiments, ATCC 39307 can be used as a reference strain.

Additional Components

In preferred embodiments, the composition further comprises one or more nutritive mineral components. In certain embodiments, the nutritive mineral component is salt (NaCl) at a concentration of 1% to 99.9%, from 15% to 98%, from 20% to 95%, from 30% to 90%, or from 50% to 80% by weight NaCl.

In certain embodiments, the nutritive mineral components include, for example, molasses, urea, proteins, and/or sources of salt, sodium, chloride, calcium, phosphorus, magnesium, potassium, phosphorous, sulfur, cobalt, copper, iron, selenium, iodine, manganese, fluorine and/or zinc.

In certain embodiments, the sources of these minerals are selected from, for example, sodium chloride, sodium molybdate, calcium carbonate, calcium iodate, calcium pantothenate, dicalcium phosphate, monocalcium phosphate, cobalt carbonate, cobalt sulfate, copper carbonate,

copper chloride, copper sulfate, amino acid chelates of copper, ethylenediamine dihydriodide, iron carbonate, red iron oxide, iron sulfate, lignin sulfonate, lime stock feed, magnesium mica, magnesium oxide, manganese sulfate, manganous oxide, mineral oil, oyster shell, potassium chloride, potassium iodate, selenium yeast, sodium selenite, zinc oxide, zinc sulfate, amino acid chelates of zinc, and others.

The concentration of each mineral component can be 0.0001 to 99% by weight, 0.0005 to 95%, 0.001 to 90%, 0.0025 to 80%, 0.005 to 70%, 0.01 to 60%, 0.025 to 50%, 0.05 to 40%, or at least: 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, 8.0%, 9.0%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%. The concentration of each mineral component can depend on factors including the nutritional needs and species of animal, and whether the composition is made available as a block, loose pellets or granules, or as a concentrate.

In certain embodiments, the composition further comprises one or more additional components, including, for example, binders, dyes, anti-caking agents, flavorings, mineral oils, vegetable oils, energy sources (e.g., molasses), preservatives, bone meal, clay, lime, amino acids (including essential amino acids), peptides, proteins, vitamins, microelements, fats, fatty acids, lipids, carbohydrates, sterols, prebiotics and enzymes. In some embodiments, the microorganisms of the composition produce and/or provide these substances.

Exemplary vitamins for use in the subject composition can include for example, vitamins A, E, K3, D3, B1, B3, B6, B12, C, biotin, folic acid, panthothenic acid, nicotinic acid, choline chloride, inositol and para-amino-benzoic acid. Other nutritive components may include, but are not limited to, antioxidants, beta-glucans, bile salt, cholesterol, carotenoids, and many others. Typical vitamins and minerals are those, for example, recommended for daily consumption and in the recommended daily amount (RDA), although precise amounts can vary. The composition would preferably include a complex of the RDA vitamins, minerals and trace minerals as well as those nutrients that have no established RDA, but have a beneficial role in healthy mammal physiology.

In certain embodiments, the composition comprises a germination enhancer for enhancing germination of spore-form microorganisms used in the microbe-based composition. In specific embodiments, the germination enhancers are amino acids, such as, for example, L-alanine and/or L-leucine. In one embodiment, the germination enhancer is manganese.

In one embodiment, the composition comprises one or more fatty acids. The fatty acids can be produced by the microorganisms of the composition, and/or produced separately and included as an additional component. In certain preferred embodiments, the fatty acid is a saturated long-chain fatty acid, having a carbon backbone of 14-20 carbons, such as, for example, myristic acid, palmitic acid or stearic acid. In some embodiments, a combination of two or more saturated long-chain fatty acids is included in the composition. In some embodiments, a saturated long-chain fatty acid can inhibit methanogenesis and/or increase cell membrane permeability of methanogens.

In some embodiments, the composition can comprise additional components known to reduce methane in the animal's digestive system, such as, for example, seaweed (e.g., *Asparagopsis taxiformis*); kelp; nitrooxypropanols (e.g., 3-nitrooxypropanol and/or ethyl-3-nitrooxypropanol); anthraquinones; ionophores (e.g., monensin and/or lasalocid); polyphenols (e.g., saponins, tannins); *Yucca schidigera* extract (steroidal saponin-producing plant species); *Quillaja saponaria* extract (triterpenoid saponin-producing plant species); organosulfurs (e.g., garlic extract); flavonoids (e.g., quercetin, rutin, kaempferol, naringin, and anthocyanidins; bioflavonoids from green citrus fruits, rose hips and black currants); carboxylic acid; and/or terpenes (e.g., d-limonene, pinene and citrus extracts).

In one embodiment, the composition can comprise one or more biosurfactants. Biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms, which are biodegradable and can be efficiently produced using selected organisms on renewable substrates. All biosurfactants are amphiphiles. They consist of two parts: a polar (hydrophilic) moiety and non-polar (hydrophobic) group. The common lipophilic moiety of a biosurfactant molecule is the hydrocarbon chain of a fatty acid, whereas the hydrophilic part is formed by ester or alcohol groups of neutral lipids, by a carboxylate group of fatty acids or amino acids (or peptides), an organic acid in the case of flavolipids, or, in the case of glycolipids, by a carbohydrate.

Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances, increase the water bioavailability of such substances, and change the properties of bacterial cell surfaces. Biosurfactants accumulate at interfaces, thus reducing interfacial tension and leading to the formation of aggregated micellar structures in solution. Safe, effective microbial biosurfactants reduce the surface and interfacial tensions between the molecules of liquids, solids, and gases. The ability of biosurfactants to form pores and destabilize biological membranes permits their use as antibacterial, antifungal, and hemolytic agents.

Biosurfactants according to the subject invention can include, for example, glycolipids, lipopeptides, flavolipids, phospholipids, fatty acid esters, and high molecular weight polymers such as lipoproteins, lipopolysaccharide-protein complexes, and polysaccharide-protein-fatty acid complexes.

In one embodiment, the biosurfactant is a glycolipid. Glycolipids can include, for example, sophorolipids, rhamnolipids, cellobiose lipids, mannosylerythritol lipids and trehalose lipids. In one embodiment, the biosurfactant is a lipopeptide. Lipopeptides can include, for example, surfactin, iturin, arthrofactin, viscosin, fengycin, and lichenysin. In certain embodiments, a mixture of biosurfactants is used.

In one embodiment, the composition comprises a biosurfactant at a concentration of 0 to 500 ppm, about 1 to 250 ppm, about 2 to 100 ppm, about 3 to 75 ppm, about 4 to 50 ppm, or about 5 to 25 ppm of the composition.

In one embodiment, the composition comprises a biosurfactant at a concentration of 0.001 to 50 g/L of ruminal fluid, or about 0.1 to 25 g/L, or about 0.5 to 20 g/L, or about 1.0 to 15 g/L, or about

1.2 to 10 g/L, or about 1.5 to 5 g/L, or about 2 to 3 g/L. In one embodiment, the biosurfactant is added in addition to the biosurfactants produced by the microorganisms of the composition. In a specific embodiment, the added biosurfactant is a sophorolipid.

In one embodiment, the biosurfactant has been purified from the fermentation medium in which it was produced. Alternatively, in one embodiment, the biosurfactant is utilized in crude form comprising fermentation broth resulting from cultivation of a biosurfactant-producing microbe. This crude form biosurfactant solution can comprise from about 0.001% to 99%, from about 25% to about 75%, from about 30% to about 70%, from about 35% to about 65%, from about 40% to about 60%, from about 45% to about 55%, or about 50% pure biosurfactant, along with residual cells and/or nutrients.

In one embodiment, the composition comprises a saponin at 1 to 10 ml/L, or 2 to 6 ml/L of ruminal fluid. Saponins are natural surfactants that are found in many plants and that exhibit similar characteristics to microbial biosurfactants, for example, self-association and interaction with biological membranes. There are three basic categories of saponins, including triterpenoid saponins, steroidal saponins, and steroidal glycoalkaloids.

Some well-known triterpenoid saponin-accumulating plant families include the *Leguminosae*, *Amaranthaceae*, *Apiaceae*, *Caryophyllaceae*, *Aquifoliaceae*, *Araliaceae*, *Cucurbitaceae*, *Berberidaceae*, *Chenopodiaceae*, *Myrsinaceae* and *Zygophyllaceae*, among many others. Quillaja and legumes such as soybeans, beans and peas are a rich source of triterpenoid saponins. The steroidal saponins are typically found in members of the *Agavaceae*, *Alliaceae*, *Asparagaceae*, *Dioscoreaceae*, *Liliaceae*, *Amaryllidaceae*, *Bromeliaceae*, *Palmae* and *Scrophulariaceae* families and accumulate in abundance in crop plants such as yam, alliums, asparagus, fenugreek, yucca and ginseng. The steroidal glycoalkaloids are commonly found in members of the *Solanaceae* family including tomato, potato, aubergines and capsicum.

In certain embodiments, a saponin-containing plant extract may reduce methane production by altering rumen pH and/or reducing protozal methanogen symbionts.

Production of Microorganisms and/or Microbial Growth By-Products

The subject invention utilizes methods for cultivation of microorganisms and production of microbial metabolites and/or other by-products of microbial growth. The subject invention further utilizes cultivation processes that are suitable for cultivation of microorganisms and production of microbial metabolites on a desired scale. These cultivation processes include, but are not limited to, submerged cultivation/fermentation, solid state fermentation (SSF), and modifications, hybrids and/or combinations thereof.

As used herein "fermentation" refers to cultivation or growth of cells under controlled conditions. The growth could be aerobic or anaerobic. In preferred embodiments, the microorganisms are grown using SSF and/or modified versions thereof.

In one embodiment, the subject invention provides materials and methods for the production of biomass (e.g., viable cellular material), extracellular metabolites, residual nutrients and/or intracellular components.

The microbe growth vessel used according to the subject invention can be any fermenter or cultivation reactor for industrial use. In one embodiment, the vessel may have functional controls/sensors or may be connected to functional controls/sensors to measure important factors in the cultivation process, such as pH, oxygen, pressure, temperature, humidity, microbial density and/or metabolite concentration.

In a further embodiment, the vessel may also be able to monitor the growth of microorganisms inside the vessel (e.g., measurement of cell number and growth phases). Alternatively, a daily sample may be taken from the vessel and subjected to enumeration by techniques known in the art, such as dilution plating technique.

In one embodiment, the method includes supplementing the cultivation with a nitrogen source. The nitrogen source can be, for example, potassium nitrate, ammonium nitrate ammonium sulfate, ammonium phosphate, ammonia, urea, and/or ammonium chloride. These nitrogen sources may be used independently or in a combination of two or more.

The method can provide oxygenation to the growing culture. One embodiment utilizes slow motion of air to remove low-oxygen containing air and introduce oxygenated air. In the case of submerged fermentation, the oxygenated air may be ambient air supplemented daily through mechanisms including impellers for mechanical agitation of liquid, and air spargers for supplying bubbles of gas to liquid for dissolution of oxygen into the liquid.

The method can further comprise supplementing the cultivation with a carbon source. The carbon source is typically a carbohydrate, such as glucose, sucrose, lactose, fructose, trehalose, mannose, mannitol, and/or maltose; organic acids such as acetic acid, fumaric acid, citric acid, propionic acid, malic acid, malonic acid, and/or pyruvic acid; alcohols such as ethanol, propanol, butanol, pentanol, hexanol, isobutanol, and/or glycerol; fats and oils such as soybean oil, canola oil, rice bran oil, olive oil, corn oil, sesame oil, and/or linseed oil; etc. These carbon sources may be used independently or in a combination of two or more.

In one embodiment, growth factors and trace nutrients for microorganisms are included in the medium. This is particularly preferred when growing microbes that are incapable of producing all of the vitamins they require. Inorganic nutrients, including trace elements such as iron, zinc, copper, manganese, molybdenum and/or cobalt may also be included in the medium. Furthermore, sources of vitamins, essential amino acids, and microelements can be included, for example, in the form of flours or meals, such as corn flour, or in the form of extracts, such as yeast extract, potato extract, beef extract, soybean extract, banana peel extract, and the like, or in purified forms. Amino acids such as, for example, those useful for biosynthesis of proteins, can also be included.

In one embodiment, inorganic salts may also be included. Usable inorganic salts can be potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, magnesium sulfate, magnesium chloride, iron sulfate, iron chloride, manganese sulfate, manganese chloride, zinc sulfate, lead chloride, copper sulfate, calcium chloride, sodium chloride, calcium carbonate, and/or sodium carbonate. These inorganic salts may be used independently or in a combination of two or more.

In one embodiment, one or more biostimulants may also be included, meaning substances that enhance the rate of growth of a microorganism. Biostimulants may be species-specific or may enhance the rate of growth of a variety of species.

In some embodiments, the method for cultivation may further comprise adding an antimicrobial in the medium before, and/or during the cultivation process.

In certain embodiments, an antibiotic can be added to a culture at low concentrations to produce microbes that are resistant to the antibiotic. The microbes that survive exposure to the antibiotic are selected and iteratively re-cultivated in the presence of progressively higher concentrations of the antibiotic to obtain a culture that is resistant to the antibiotic. This can be performed in a laboratory setting or industrial scale using methods known in the microbiological arts. In certain embodiments, the amount of antibiotic in the culture begins at, for example, 0.0001 ppm and increases by about 0.001 to 0.1 ppm each iteration until the concentration in the culture is equal to, or about equal to, the dosage that would typically be applied to a livestock animal.

In certain embodiments, the antibiotics are those often used in livestock feed to promote growth and to help treat and prevent illness and infection in animals, such as, for example, procaine, penicillin, tetracyclines (e.g., chlortetracycline, oxytetracycline), tylosin, bacitracin, neomycin sulfate, streptomycin, erythromycin, monensin, roxarsone, salinomycin, tylosin, lincomycin, carbadox, laidlomycin, lasalocid, oleandomycin, virginamycin, and bambermycins. By producing beneficial microbes that are resistant to a particular livestock antibiotic, the microbes can be selected based on which antibiotic may be administered to the animal to treat or prevent a condition. Alternatively, an antibiotic can be selected for a livestock animal based on which beneficial microbe is being administered to the animal according to the subject methods so as not to harm the beneficial microbe.

The pH of the mixture should be suitable for the microorganism of interest. Buffers, and pH regulators, such as carbonates and phosphates, may be used to stabilize pH near a preferred value. When metal ions are present in high concentrations, use of a chelating agent in the medium may be necessary.

The microbes can be grown in planktonic form or as biofilm. In the case of biofilm, the vessel may have within it a substrate upon which the microbes can be grown in a biofilm state. The system may also have, for example, the capacity to apply stimuli (such as shear stress) that encourages and/or improves the biofilm growth characteristics.

In one embodiment, the method for cultivation of microorganisms is carried out at about 5° to about 100° C, preferably, 15 to 60° C, more preferably, 25 to 50° C. In a further embodiment, the cultivation may be carried out continuously at a constant temperature. In another embodiment, the cultivation may be subject to changing temperatures.

In one embodiment, the equipment used in the method and cultivation process is sterile. The cultivation equipment such as the reactor/vessel may be separated from, but connected to, a sterilizing unit, e.g., an autoclave. The cultivation equipment may also have a sterilizing unit that sterilizes *in situ* before starting the inoculation. Air can be sterilized by methods know in the art. For example, the ambient air can pass through at least one filter before being introduced into the vessel. In other embodiments, the medium may be pasteurized or, optionally, no heat at all added, where the use of low water activity and low pH may be exploited to control undesirable bacterial growth.

In one embodiment, the subject invention further provides a method for producing microbial metabolites such as, for example, biosurfactants, enzymes, proteins, ethanol, lactic acid, beta-glucan, peptides, metabolic intermediates, polyunsaturated fatty acid, and lipids, by cultivating a microbe strain of the subject invention under conditions appropriate for growth and metabolite production; and, optionally, purifying the metabolite. The metabolite content produced by the method can be, for example, at least 20%, 30%, 40%, 50%, 60%, 70 %, 80 %, or 90%.

The biomass content of the fermentation medium may be, for example, from 5 g/l to 180 g/l or more, or from 10 g/l to 150 g/l. The cell concentration may be, for example, at least 1 x 10^9, 1 x 10^{10}, 1 x 10^{11}, 1 x 10^{12} or 1 x 10^{13} cells per gram of final product.

The microbial growth by-product produced by microorganisms of interest may be retained in the microorganisms or secreted into the growth medium. The medium may contain compounds that stabilize the activity of microbial growth by-product.

The method and equipment for cultivation of microorganisms and production of the microbial by-products can be performed in a batch, a quasi-continuous process, or a continuous process.

In one embodiment, all of the microbial cultivation composition is removed upon the completion of the cultivation (e.g., upon, for example, achieving a desired cell density, or density of a specified metabolite). In this batch procedure, an entirely new batch is initiated upon harvesting of the first batch.

In another embodiment, only a portion of the fermentation product is removed at any one time. In this embodiment, biomass with viable cells, spores, conidia, hyphae and/or mycelia remains in the vessel as an inoculant for a new cultivation batch. The composition that is removed can be a cell-free medium or contain cells, spores, or other reproductive propagules, and/or a combination of thereof. In this manner, a quasi-continuous system is created.

Advantageously, the method does not require complicated equipment or high energy consumption. The microorganisms of interest can be cultivated at small or large scale on site and utilized, even being still-mixed with their media.

Local Production of Microbe-Based Products

A "microbe-based product," is a product to be applied in practice to achieve a desired result. The microbe-based product can be simply a microbe-based composition harvested from a microbe cultivation process. Alternatively, a microbe-based product may comprise further ingredients that have been added. These additional ingredients can include, for example, stabilizers, buffers, carriers (e.g., water or salt solutions), added nutrients to support further microbial growth, non-nutrient growth enhancers and/or agents that facilitate tracking of the microbes and/or the composition in the environment to which it is applied. The microbe-based product may also comprise mixtures of microbe-based compositions. The microbe-based product may also comprise one or more components of a microbe-based composition that have been processed in some way such as, but not limited to, filtering, centrifugation, lysing, drying, purification and the like.

One microbe-based product of the subject invention is simply the fermentation medium containing a microorganism and/or the microbial metabolites produced by the microorganism and/or any residual nutrients. The product of fermentation may be used directly without extraction or purification. If desired, extraction and purification can be easily achieved using standard extraction and/or purification methods or techniques described in the literature.

The microorganisms in the microbe-based product may be in an active or inactive form. Furthermore, the microorganisms may be removed from the composition, and the residual culture utilized. The microbe-based products may be used without further stabilization, preservation, and storage. Advantageously, direct usage of these microbe-based products preserves a high viability of the microorganisms, reduces the possibility of contamination from foreign agents and undesirable microorganisms, and maintains the activity of the by-products of microbial growth.

The microbes and/or medium (e.g., broth or solid substrate) resulting from the microbial growth can be removed from the growth vessel and transferred via, for example, piping for immediate use.

In one embodiment, the microbe-based product is simply the growth by-products of the microorganism. For example, biosurfactants produced by a microorganism can be collected from a submerged fermentation vessel in crude form, comprising, for example about 50% pure biosurfactant in liquid broth.

In other embodiments, the microbe-based product (microbes, medium, or microbes and medium) can be placed in containers of appropriate size, taking into consideration, for example, the intended use, the contemplated method of application, the size of the fermentation vessel, and any mode of transportation from microbe growth facility to the location of use. Thus, the containers into which the microbe-based composition is placed may be, for example, from 1 gallon to 1,000 gallons or more. In other embodiments the containers are 2 gallons, 5 gallons, 25 gallons, or larger.

Upon harvesting, for example, the yeast fermentation product, from the growth vessels, further components can be added as the harvested product is placed into containers and/or piped (or

otherwise transported for use). The additives can be, for example, buffers, carriers, other microbe-based compositions produced at the same or different facility, viscosity modifiers, preservatives, nutrients for microbe growth, tracking agents, solvents, biocides, other microbes and other ingredients specific for an intended use.

Other suitable additives, which may be contained in the formulations according to the invention, include substances that are customarily used for such preparations. Examples of such additives include surfactants, emulsifying agents, lubricants, buffering agents, solubility controlling agents, pH adjusting agents, preservatives, stabilizers and ultra-violet light resistant agents.

In one embodiment, the product may further comprise buffering agents including organic and amino acids or their salts. Suitable buffers include citrate, gluconate, tartarate, malate, acetate, lactate, oxalate, aspartate, malonate, glucoheptonate, pyruvate, galactarate, glucarate, tartronate, glutamate, glycine, lysine, glutamine, methionine, cysteine, arginine and a mixture thereof. Phosphoric and phosphorous acids or their salts may also be used. Synthetic buffers are suitable to be used but it is preferable to use natural buffers such as organic and amino acids or their salts listed above.

In a further embodiment, pH adjusting agents include potassium hydroxide, ammonium hydroxide, potassium carbonate or bicarbonate, hydrochloric acid, nitric acid, sulfuric acid or a mixture.

Advantageously, in accordance with the subject invention, the microbe-based product may comprise broth in which the microbes were grown. The product may be, for example, at least, by weight, 1%, 5%, 10%, 25%, 50%, 75%, or 100% broth. The amount of biomass in the product, by weight, may be, for example, anywhere from 0% to 100% inclusive of all percentages therebetween.

In certain embodiments of the subject invention, a microbe growth facility produces fresh, high-density microorganisms and/or microbial growth by-products of interest on a desired scale. The microbe growth facility may be located at or near the site of application. The facility produces high-density microbe-based compositions in batch, quasi-continuous, or continuous cultivation.

The microbe growth facilities of the subject invention can be located at the location where the microbe-based product will be used (e.g., a free-range cattle pasture). For example, the microbe growth facility may be less than 300, 250, 200, 150, 100, 75, 50, 25, 15, 10, 5, 3, or 1 mile from the location of use.

Because the microbe-based product can be generated locally, without resort to the microorganism stabilization, preservation, storage and transportation processes of conventional microbial production, a much higher density of microorganisms can be generated, thereby requiring a smaller volume of the microbe-based product for use in the on-site application or which allows much higher density microbial applications where necessary to achieve the desired efficacy. This allows for a scaled-down bioreactor (e.g., smaller fermentation vessel, smaller supplies of starter material, nutrients and pH control agents), which makes the system efficient and can eliminate the need to stabilize cells or separate them from their culture medium. Local generation of the microbe-based

product also facilitates the inclusion of the growth medium in the product. The medium can contain agents produced during the fermentation that are particularly well-suited for local use.

Locally-produced high density, robust cultures of microbes are more effective in the field than those that have remained in the supply chain for some time. The microbe-based products of the subject invention are particularly advantageous compared to traditional products wherein cells have been separated from metabolites and nutrients present in the fermentation growth media. Reduced transportation times allow for the production and delivery of fresh batches of microbes and/or their metabolites at the time and volume as required by local demand.

The microbe growth facilities of the subject invention produce fresh, microbe-based compositions, comprising the microbes themselves, microbial metabolites, and/or other components of the medium in which the microbes are grown. If desired, the compositions can have a high density of vegetative cells or propagules, or a mixture of vegetative cells and propagules.

In one embodiment, the microbe growth facility is located on, or near, a site where the microbe-based products will be used (e.g., a livestock production facility), preferably within 300 miles, more preferably within 200 miles, even more preferably within 100 miles. Advantageously, this allows for the compositions to be tailored for use at a specified location. The formula and potency of microbe-based compositions can be customized for specific local conditions at the time of application, such as, for example, which animal species is being treated; what season, climate and/or time of year it is when a composition is being applied; and what mode and/or rate of application is being utilized.

Advantageously, distributed microbe growth facilities provide a solution to the current problem of relying on far-flung industrial-sized producers whose product quality suffers due to upstream processing delays, supply chain bottlenecks, improper storage, and other contingencies that inhibit the timely delivery and application of, for example, a viable, high cell-count product and the associated medium and metabolites in which the cells are originally grown.

Furthermore, by producing a composition locally, the formulation and potency can be adjusted in real time to a specific location and the conditions present at the time of application. This provides advantages over compositions that are pre-made in a central location and have, for example, set ratios and formulations that may not be optimal for a given location.

The microbe growth facilities provide manufacturing versatility by their ability to tailor the microbe-based products to improve synergies with destination geographies. Advantageously, in preferred embodiments, the systems of the subject invention harness the power of naturally-occurring local microorganisms and their metabolic by-products to improve GHG management.

The cultivation time for the individual vessels may be, for example, from 1 to 7 days or longer. The cultivation product can be harvested in any of a number of different ways.

Local production and delivery within, for example, 24 hours of fermentation results in pure, high cell density compositions and substantially lower shipping costs. Given the prospects for rapid

advancement in the development of more effective and powerful microbial inoculants, consumers will benefit greatly from this ability to rapidly deliver microbe-based products.

Preparation of Compressed Mineral Assemblies

Methods known in the art for producing compressed mineral assemblies can be used, including pressurized molding, extrusion, and/or pelleting.

In an exemplary embodiment, compressed mineral assemblies may be prepared by, e.g., extrusion, which includes mixing, cooking, shaping and cutting raw ingredients into a specific shape and size in a very short period of time. The ingredients may be mixed into homogenous expandable dough and cooked in an extruder, and forced through a die under pressure and high heat. After cooking, the shapes are then allowed to cool. The dough can also be poured into a mold and pressed at high pressure and/or dried using hot air.

In one embodiment, a "cold" process can be used, or a process that does not use high heat or steam. The process can use, for example, liquid binders with viscous and cohesive properties to hold the ingredients together without risk of denaturing or degrading important components and/or nutrients in the compositions of the subject invention.

In another embodiment, the composition can be mixed with a livestock animal's feed or drinking water as, for example, a concentrate. The dosage of the concentrate should be adjusted so that the mineral components are diluted to a safe level upon mixing with the feed or water.

Methods for Reducing Greenhouse Gas Emissions

In preferred embodiments, the subject invention provides methods of feeding an animal, wherein a digestive health composition according to the subject invention is made available to the animal such that the animal can ingest the composition.

In preferred embodiments, the composition is made available to the livestock in the form of a mineral block, salt lick, lick block, multi-nutrient block, molasses block, UMMB, HQFB, multi-species block, protein block, granules, crystals or pellets, which, when placed in a location where the animal grazes, feeds or traverses, can be licked or chewed by the animal upon the animal's choosing.

As is known in the agricultural arts, the composition is preferably made available in proximity (e.g., less than 1,000 feet) to a source of drinking water.

In some embodiments, the composition is made available in the form of granules, pellets or a concentrate that are mixed with the animal's feed and/or drinking water at a pre-determined dosage.

"Livestock" animals, as used herein, are "domesticated" animals, meaning species that have been influenced, bred, tamed, and/or controlled over a sustained number of generations by humans, such that a mutualistic relationship exists between the animal and the human. Particularly, livestock animals include animals raised in an agricultural or industrial setting to produce commodities such as food, fiber and labor. Types of animals included in the term livestock can include, but are not limited

to, alpacas, llamas, pigs (swine), horses, mules, asses, camels, dogs, ruminants, chickens, turkeys, ducks, geese, guinea fowl, and squabs.

In certain embodiments, the livestock animals are "ruminants," or mammals that utilize a compartmentalized stomach suited for fermenting plant-based foods prior to digestion with the help of a specialized gut microbiome. Ruminants include, for example, bovines, sheep, goats, ibex, giraffes, deer, elk, moose, caribou, reindeer, antelope, gazelle, impala, wildebeest, and some kangaroos.

In specific exemplary embodiments, the livestock animals are bovine animals, which are ruminant animals belonging to the subfamily Bovinae, of the family Bovidae. Bovine animals can include domesticated and/or wild species. Specific examples include, but are not limited to, water buffalo, anoa, tamaraw, auroch, banteng, guar, gayal, yak, kouprey, domestic meat and dairy cattle (e.g., *Bos taurus*, *Bos indicus*), ox, bullock, zebu, saola, bison, buffalo, wisent, bongo, kudu, kewwel, imbabala, kudu, nyala, sitatunga, and eland.

Wild animals, such as deer, bears, birds, foxes, bison, water buffalo, monkeys, apes, elephants, tigers and lions can also benefit from the compositions and methods of subjectivation.

In certain embodiments, the methods enhance the livestock animal's health by supplementing its diet with salt or another trace mineral. Thus, in some embodiments, the methods can be used to treat and/or prevent salt or other mineral deficiencies, as well as conditions caused by such deficiencies (e.g., weight loss, pica, and/or decreased milk production).

In certain embodiments, the methods enhance the livestock animal's health by, for example, contributing to a healthy gut microbiome; improving digestion; increasing feed-to-muscle conversion ratio; increasing milk production and quality; modulating the immune system; and/or increasing life expectancy.

In certain embodiments, the methods reduce methane, carbon dioxide and/or other deleterious atmospheric gases, and/or precursors thereof (e.g., nitrogen and/or ammonia, which are precursors of nitrous oxide) that are typically produced in the digestive system and/or waste of livestock animals.

As used herein, "reduction" refers to a negative alteration of at least 0.25%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

In some embodiments, the desired reduction is achieved within a relatively short time period, for example, within 1 week, 2 weeks, 3 weeks or 4 weeks of the animals ingesting the composition. In some embodiments, the desired reduction is achieved within, for example, 1 month, 2 months, 3 months, 4 months, 5 months or 6 months after employing the subject methods. In some embodiments, the desired reduction is achieved within 1 year, 2 years, 3 years, 4 years, or 5 years after employing the subject methods.

Advantageously, in preferred embodiments, the methods can result in a direct inhibition of methanogenic bacteria and/or symbionts thereof, disruption of methanogenic biofilms, and/or

disruption of the biological pathway involved in methanogenesis in the livestock animal's digestion system, for example, the rumen, stomach and/or intestines.

In certain embodiments, the methods can also counteract H_2-acceptor depletion that results from reduced methanogenesis. Accordingly, potential negative effects of excessive H_2 on livestock products can be prevented and/or reduced. For example, excess H_2 in the digestive tract of mammals can produce a fishy smell in milk due to the overproduction of trimethylamine.

In some embodiments, the methods result in increased conversion of nitrogen to muscle mass, thereby reducing the amount of nitrogen that is available for production of ammonia and nitrous oxide.

In some embodiments, the beneficial microorganisms of the composition can survive transport through the digestive system and are excreted with the animal's waste, where they continue inhibiting methanogens and/or symbionts thereof, disrupting methanogenic biofilms, disrupting the biological pathways involved in methanogenesis, and/or compensating for H_2 acceptor loss.

In some embodiments, prior to making the composition available to the livestock animal, the method comprises assessing a livestock animal, herd of livestock animals, or livestock waste storage site for local conditions, determining a preferred formulation for the composition (e.g., the type, combination and/or ratios of microorganisms and/or growth by-products) that is customized for the local conditions, and producing the composition with said preferred formulation.

The local conditions can include, for example, age, health, size and species of the animal(s); herd size; purpose for producing the animal (e.g., meat, fur, fiber, labor, milk, etc.); species within the microbial population of an animal's gut and/or waste; environmental conditions, such as amount and type of GHG emissions, current climate, and/or season/time of year; mode and/or rate of application of the composition, and others as are deemed relevant.

After assessment, a preferred formulation for the composition can be determined so that the composition can be customized for these local conditions. The composition is then cultivated, preferably at a microbe growth facility that is within 300 miles, preferably within 200 miles, even more preferably within 100 miles of the location of application (e.g., an animal or livestock production facility, or a lagoon).

In some embodiments the local conditions are assessed periodically, for example, once annually, biannually, or even monthly. In this way, the composition formula can be modified in real time as necessary to meet the needs of the changing local conditions.

In an exemplary embodiment, the daily dosage of a microorganism of the subject invention that is administered to each animal is about 10 mg to about 10 g, or about 15 mg to about 5 grams, per 100 kg of animal body weight.

According to the methods of the subject invention, administration of the microbe-based compositions can be performed as part of a dietary regimen, which can span a period ranging from parturition through the adult life of the animal. In certain embodiments, the animal is a young or

growing animal. In some embodiments, the animal is an aging animal. In other embodiments administration begins, for example, on a regular or extended regular basis, when the animal has reached more than about 30%, 40%, 50%, 60%, or 80% of its projected or anticipated lifespan.

In some embodiments, the methods of the subject invention can be utilized by a livestock producer or waste processor for reducing carbon credit usage. Thus, in certain embodiments, the subject methods can further comprise conducting measurements to assess the effect of the method on reducing the generation of carbon dioxide and/or other deleterious atmospheric gases, and/or precursors thereof (e.g., nitrogen and/or ammonia), and/or to assess the effect of the method on the control of methanogens in the livestock animal's digestive system and/or waste, using standard techniques in the art.

These measurements can be conducted according to known methods in the art (*see, e.g.*, Storm et al. 2012, incorporated herein by reference), including, for example, gas capture and quantification, chromatography, respiration chambers (which measure the amount of methane exhaled by an individual animal), and *in vitro* gas production technique (where feed is fermented under controlled laboratory and microbial conditions to determine amount of methane and/or nitrous oxide is emitted per gram of dry matter). The measurements can also come in the form of testing the microbial population in an animal, for example, by sampling milk, feces, and/or stomach contents and using, for example, DNA sequencing and/or cell plating to determine the number of methanogenic microbes present therein.

Measurements can be conducted at a certain time point after application of the microbe-based composition. In some embodiments, the measurements are conducted after about 1 week or less, 2 weeks or less, 3 weeks or less, 4 weeks or less, 30 days or less, 60 days or less, 90 days or less, 120 days or less, 180 days or less, and/or 1 year or less.

Furthermore, the measurements can be repeated over time. In some embodiments, the measurements are repeated daily, weekly, monthly, bi-monthly, semi-monthly, semi-annually, and/or annually.

EXAMPLES

A greater understanding of the present invention and of its many advantages may be had from the following examples, given by way of illustration. The following examples are illustrative of some of the methods, applications, embodiments and variants of the present invention. They are not to be considered as limiting the invention. Numerous changes and modifications can be made with respect to the invention.

EXAMPLE 1 – IN VITRO TESTING

Compositions according to embodiments of the subject invention were screened for their ability to reduce enteric methane and carbon dioxide emissions in cattle. Twenty-four vessels were filled with cattle rumen fluid, artificial saliva, 1g rumen solids, 1g super basic ration and 1% by

volume of a treatment composition. Triplicates of eight treatments were performed, including one control triplicate.

Treatments included:

0 – Control

1 – *B. amy*

2 – *P. ostreatus*

3 – *S. boulardii*

4 – *B. amy* + *P. ostreatus*

5 – *B. amy* + *S. boulardii*

6 – *P. ostreatus* + *S. boulardii*

7 – *B. amy* + *P. ostreatus* + *S. boulardii*

After 24 hours, the amount of methane, carbon dioxide and total gas volumes (ml/gDM) collected from each vessel was measured.

FIG. 2 shows the results for methane. Treatment 1, comprising *B. amy*, showed a 78% reduction ($p = 0.05$) in average amount of methane gas compared to the control. Treatment 6, comprising *S. boulardii* and *P. ostreatus*, showed a 69% reduction ($p = 0.03$) in average amount of methane gas compared to the control.

FIG. 3 shows the results for carbon dioxide reduction. Treatment 1, comprising *B. amy*, showed the greatest reduction in average amount of carbon dioxide gas compared to the control, and Treatment 6, comprising *S. boulardii* and *P. ostreatus*, showed the next greatest reduction.

EXAMPLE 2 – ADDITIONAL IN VITRO TESTING

Treatment #1 from Example 1 above comprising *B. amy* was screened at variable inclusion rates for its ability to reduce enteric methane and carbon dioxide emissions in cattle. Eight replicates each of five different inclusion rates were conducted in individual vessels (equaling 40 vessels total). The vessels comprised rumen fluid, artificial saliva, 1 g rumen solids, 1 g super basic ration, and a variable inclusion rate of Treatment #1. The variable inclusion rates were: 0% (control), 0.1%, 0.2%, 0.5% and 1%.

Twenty-four hours after initiation of in-vitro rumen fermentation, the amount of methane, carbon dioxide and total gas volumes (ml/gDM) collected from each vessel was measured.

FIG. 4 shows the results for methane. The treatment comprising an inclusion rate of 0.2% *B. amy*, showed the greatest reduction in CH_4 emissions. (* indicates a significant reduction, $p = 0.0174$).

FIG. 5 shows the results for carbon dioxide. The treatment comprising an inclusion rate of 0.2% *B. amy*, showed the greatest reduction in CO_2 emissions. (* indicates a significant reduction, $p = 0.0491$).

EXAMPLE 3 – *B. AMY* PRODUCT

One microbe-based product of the subject invention comprises *B. amy*. *B. amy* inoculum is grown in a small-scale reactor for 24 to 48 hours. *Myxococcus xanthus* inoculum is grown in a 2L working volume seed culture flask for 48 to 120 hours. A fermentation reactor is inoculated with the two inocula. Nutrient medium is fed to the fermentation reactor continuously from a feed tank. The nutrient medium comprises:

Glucose	1 g/L to 5 g/L
Casein peptone	1 g/L to 10 g/L
K_2HPO_4	0.01 g/L to 1.0 g/L
KH_2PO_4	0.01 g/L to 1.0 g/L
$MgSO_4.7H_2O$	0.01 g/L to 1.0 g/L
NaCl	0.01 g/L to 1.0 g/L
$CaCO_3$	0.5 g/L to 5 g/L
$Ca(NO_3)_2$	0.01 g/L to 1.0 g/L
Yeast extract	0.01 g/L to 5 g/L
$MnCl_2.4H_2O$	0.001 g/L to 0.5 g/L
Teknova trace element	0.5 ml/L to 5 ml/L

Fine grain particulate anchoring carrier is suspended in the nutrient medium. The carrier comprises cellulose (1.0 to 5.0 g/L) and/or corn flour (1.0 to 8.0 g/L).

pH in the reactor is maintained at about 6.8; temperature is maintained at about 24°C; DO is maintained at about 50%; and air flow rate is maintained at about 1 vvm.

A foam layer comprising microbial growth by-products is produced during fermentation and is purged out and collected in a container comprising a pH meter. The pH meter is used to monitor the pH of the foam: if the pH varies outside of the range of 2.0 to 3.0, pH adjusters are added to bring the pH back within that range for long-term preservation of the metabolites therein. Foam continues to be produced, purged from the reactor, and collected for 7 days or longer (e.g., indefinitely).

Sampling of the fermenter and the foam collection tank for CFU count, sporulation percentage and/or purity is performed at 0 hr., then twice per day throughout fermentation. Sampling can also occur at the time that foam is purged and collected. When/if sporulation percentage of the bacterial culture is detected (using microscope slide estimation) to be greater than 20%, additional nutrient media is added to the fermenter. LC-MS analysis is carried out on acidified lipopeptide samples from the foam collection tank. The samples are stored at about 4°C.

The fermentation cycle is continued for at least one week, with nutrient medium feeding and foam collection occurring until, for example, foam can no longer be extracted from the fermenter. Lipopeptide production is observed in as little as 3 hours after inoculation, with a total yield reaching 20 to 30 g/L per week (or 250 dry kg of lipopeptide per week). The yield from this method can reach up to 10 times greater than traditional, non-antagonistic methods of cultivation *B. amyloliquefaciens.*

Concentration and drying of product

The cell biomass, comprising *B. amy* spores, is collected and dried to a residual moisture no higher than 8%. The remaining cell-free foam and/or supernatant, which can reduce surface tension to 29-30 mN/m at 200ppm, is evaporated using industrial evaporators to obtain a highly-viscous liquid containing biosurfactants and other metabolites. The viscous compound is then dried to produce a powder, which is milled and mixed with the dry spores at a ratio of 1 g to 50 mg, spores to supernatant.

The final product preferably contains no less than 100 billion spores per gram. The ideal treatment for cattle is 1 g of the composition per head of cattle per day, or if applied to a pasture, 1 g per 100 sq. feet of pasture per week.

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CLAIMS

We claim:

1. A method for feeding an animal, the method comprising making a digestive health composition available to the animal such that the animal can ingest the digestive health composition, wherein the digestive health composition comprises one or more beneficial microorganisms and/or growth by-products thereof, and one or more nutritive mineral components, wherein the digestive health composition is formulated as a compressed nutrient and/or mineral block, granule, crystal, or powder, and wherein the animal ingests the composition via licking or chewing.

2. The method of claim 1, wherein the one or more beneficial microorganisms are *Bacillus* spp. bacteria.

3. The method of claim 2, wherein the *Bacillus* spp. bacteria are selected from *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis*.

4. The method of claim 2, wherein the *Bacillus* sp. bacterium is *B. amyloliquefaciens* NRRL B-67928 (strain *B. amy*).

5. The method of claim 2, wherein the *Bacillus* sp. bacterium is *B. subtilis* NRRL B-68031 (strain B4).

6. The method of claim 2, comprising strain *B. amy* and strain B4.

7. The method of claim 1, wherein the one or more microbial growth by-products are selected from biosurfactants, enzymes, organic acids, fatty acids, amino acids, proteins, peptides, alcohols, statins, polyketides, natural antibiotics, aldehydes, amines, sterols and vitamins.

8. The method of claim 1, wherein the nutritive mineral components are selected from sodium, chloride, calcium, magnesium, phosphorus, potassium, sulfur, chromium, cobalt, copper, iodine, iron, manganese, molybdenum, selenium, and zinc.

9. The method of claim 1, wherein the digestive health composition further comprises nutrients to supplement the animal's nutritional needs and promote health and/or well-being in the animal, wherein said nutrients are sources of amino acids, peptides, proteins, vitamins, microelements, fats, fatty acids, lipids, carbohydrates, sterols, antioxidants and/or enzymes.

10. The method of claim 9, wherein the fatty acids are selected from stearic acid, palmitic acid and myristic acid.

11. The method of claim 1, wherein the digestive health composition further comprises a germination enhancer selected from L-alanine, L-leucine and manganese.

12. The method of claim 1, wherein the digestive health composition further comprises one or more of the following components: seaweed (*Asparagopsis taxiformis*); kelp; 3-nitrooxypropanol; anthraquinones; ionophores selected from monensin and lasalocid; polyphenols selected from saponins and tannins; *Yucca schidigera* extract (steroidal saponin-producer); *Quillaja saponaria* extract (triterpenoid saponin-producing plant species); organosulfurs; garlic extract; flavonoids selected from quercetin, rutin, kaempferol, naringin, and anthocyanidins; bioflavonoids isolated from green citrus fruits, rose hips and/or black currants; carboxylic acid; and terpenes selected from d-limonene, pinene and citrus extracts.

13. The method of claim 1, wherein the health of the animal is enhanced by supplementing the animal's diet with salt or another necessary mineral.

14. The method of claim 1, used to treat and/or prevent a mineral deficiency in an animal.

15. The method of claim 1, wherein the health of the animal is enhanced by, for example, improving the animal's gut microbiome; improving the animal's digestion; increasing feed-to-muscle conversion ratio in the animals; increasing the animal's milk production and quality of milk; modulating the animal's immune system; and/or increasing the animal's life expectancy.

16. The method of claim 1, wherein emissions of deleterious atmospheric gases and/or precursors thereof are reduced from the animal's digestive processes and/or waste.

17. The method of claim 16, wherein the deleterious atmospheric gases are methane, carbon dioxide and nitrous oxide.

18. The method of claim 16, wherein the deleterious atmospheric gas precursors are nitrogen and ammonia.

19 The method of claim 1, wherein a methanogenic bacterium and/or a protozoan in the livestock animal's digestive system is controlled.

20. The method of claim 1, used for reducing the number of carbon credits used by an operator involved in livestock production.

21. The method of any of claims 1 to 20, wherein the animal is a livestock animal.

22. A composition for feeding an animal, the composition comprising one or more beneficial microorganisms and/or growth by-products thereof, and one or more nutritive mineral components.

23. The composition of claim 22, wherein the beneficial microorganism is a strain of *Bacillus*.

24. The composition of claim 23, wherein the strain of *Bacillus* is *B. amyloliquefaciens* NRRL B-67928 (strain *B. amy*).

25. The composition of claim 23, wherein the strain of *Bacillus* is *B. subtilis* NRRL B-68031 (strain B4).

26. The composition of claim 22, wherein the nutritive mineral components are selected from sodium, chloride, calcium, magnesium, phosphorus, potassium, sulfur, chromium, cobalt, copper, iodine, iron, manganese, molybdenum, selenium, and zinc.

27. The composition of claim 22, formulated as a compressed mineral block, granule, crystal, or powder.

28. The composition of claim 27, formulated as a salt lick.

29. The composition of claim 22, further comprising non-mineral nutrients to supplement the livestock animal's nutritional needs and promote health and/or well-being in the livestock animal, wherein said nutrients are selected from sources molasses, urea, and sources of amino acids, peptides, proteins, vitamins, microelements, fats, fatty acids, lipids, carbohydrates, sterols, antioxidants and enzymes.

30. The composition of claim 22, further comprising a saturated long chain fatty acid selected from stearic acid, palmitic acid and myristic acid.

31. The composition of claim 22, further comprising a germination enhancer selected from L-alanine, L-leucine and manganese.

32. The composition of claim 22, further comprising one or more of the following components: seaweed (*Asparagopsis taxiformis*); kelp; nitrooxypropanols (e.g., 3-nitrooxypropanol and/or ethyl-3-nitrooxypropanol); anthraquinones; ionophores selected from monensin and lasalocid; polyphenols selected from saponins and tannins; *Yucca schidigera* extract (steroidal saponin-producer); *Quillaja saponaria* extract (triterpenoid saponin-producing plant species); organosulfurs; garlic extract; flavonoids selected from quercetin, rutin, kaempferol, naringin, and anthocyanidins; bioflavonoids isolated from green citrus fruits, rose hips and/or black currants; carboxylic acid; and terpenes selected from d-limonene, pinene and citrus extracts.

33. The composition of claim 20, wherein the one or more microbial growth by-products are selected from biosurfactants, enzymes, organic acids, fatty acids, amino acids, proteins, peptides, alcohols, statins, polyketides, natural antibiotics, aldehydes, amines, sterols and vitamins.

34. A method for reducing greenhouse gas emissions from livestock animals and/or livestock animals' waste, the method comprising making a composition according to any one of claims 22-33 available to a livestock animal such that the livestock animal can ingest the composition, and allowing the animal to ingest the composition via licking or chewing.

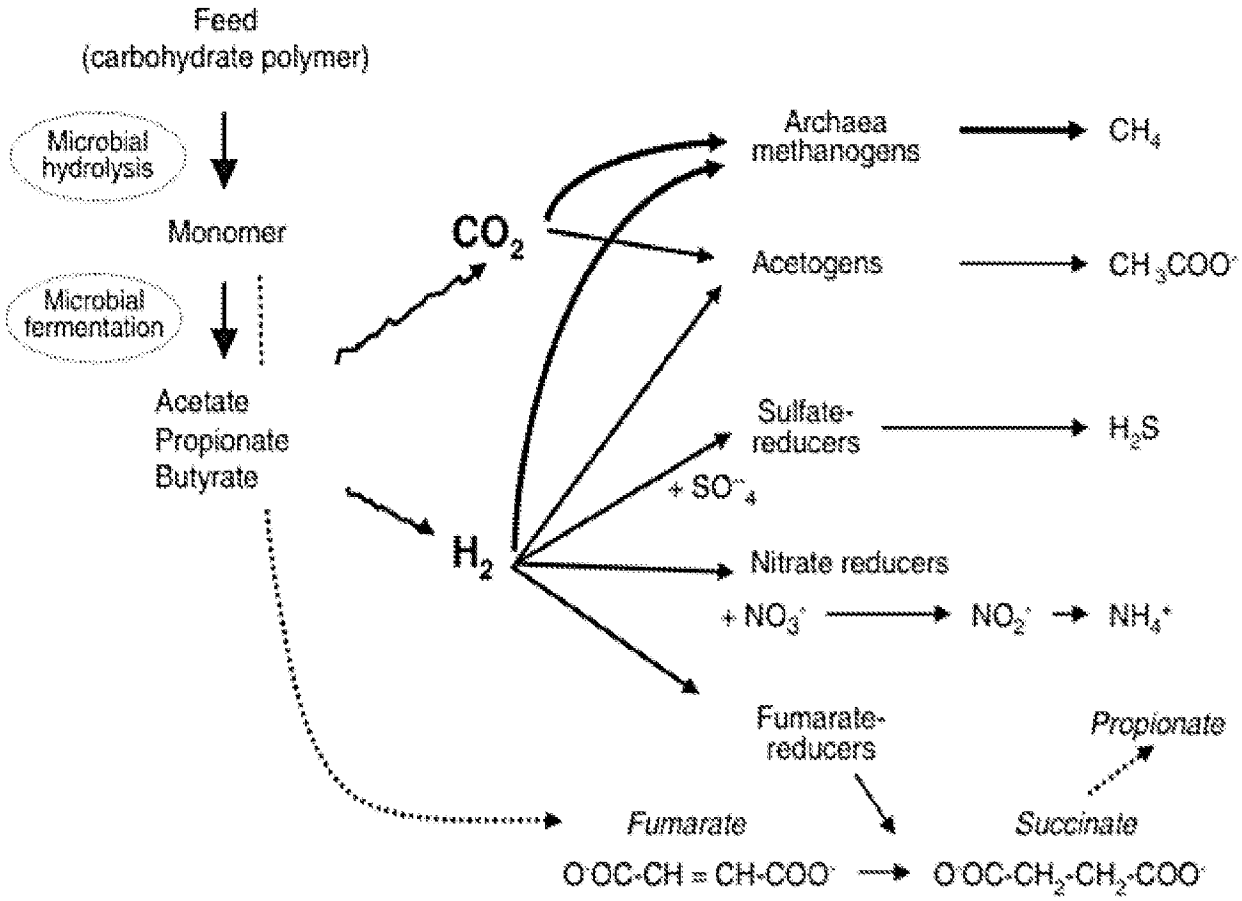
Feed
(carbohydrate polymer)
Microbial hydrolysis
Monomer
Microbial fermentation
Acetate
Propionate
Butyrate
CO2
H2
Archaea methanogens
CH4
Acetogens
CH3COO-
Sulfate-reducers
H2S
+ SO4--
Nitrate reducers
+ NO3- NO2- NH4+
Fumarate-reducers
Propionate
Fumarate
Succinate
O-OC-CH = CH-COO-
O-OC-CH2-CH2-COO-

FIG. 1

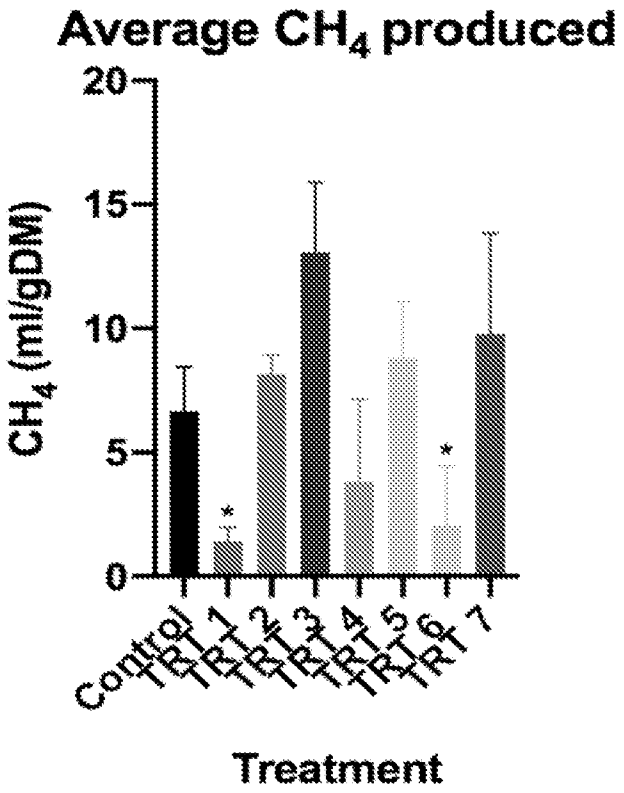
Average CH_4 produced
CH_4 (ml/gDM)
20
15
10
5
0
*
*
Control
TRT 1
TRT 2
TRT 3
TRT 4
TRT 5
TRT 6
TRT 7
Treatment

FIG. 2

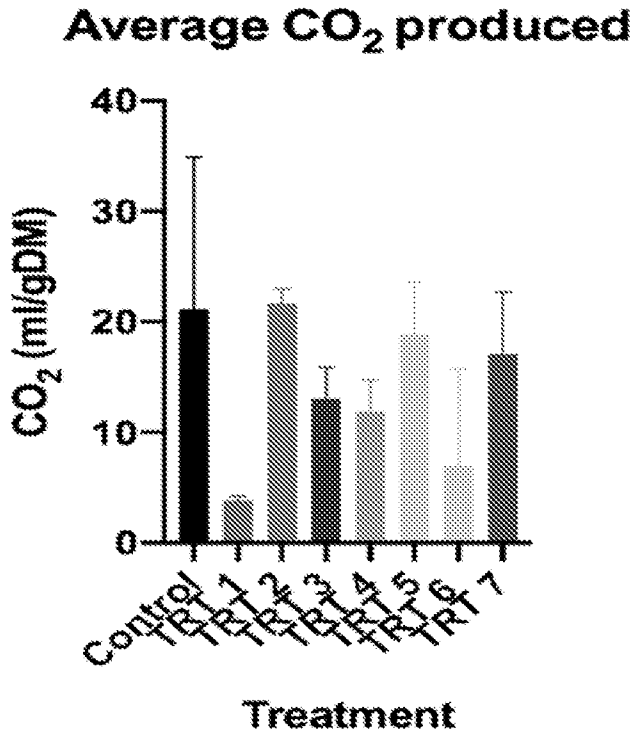
Average CO_2 produced
CO_2 (ml/gDM)
40
30
20
10
0
Control
TRT 1
TRT 2
TRT 3
TRT 4
TRT 5
TRT 6
TRT 7
Treatment

FIG. 3

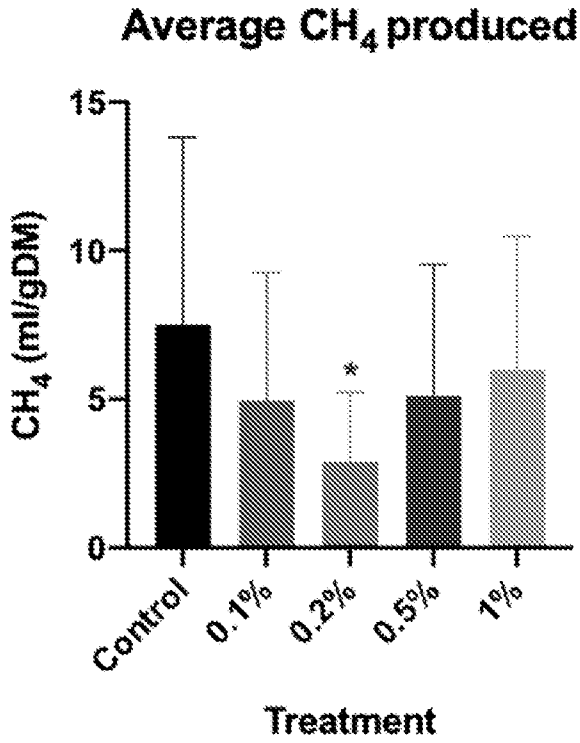
Average CH_4 produced
15
10
5
0
CH_4 (ml/gDM)
*
Control
0.1%
0.2%
0.5%
1%
Treatment

FIG. 4

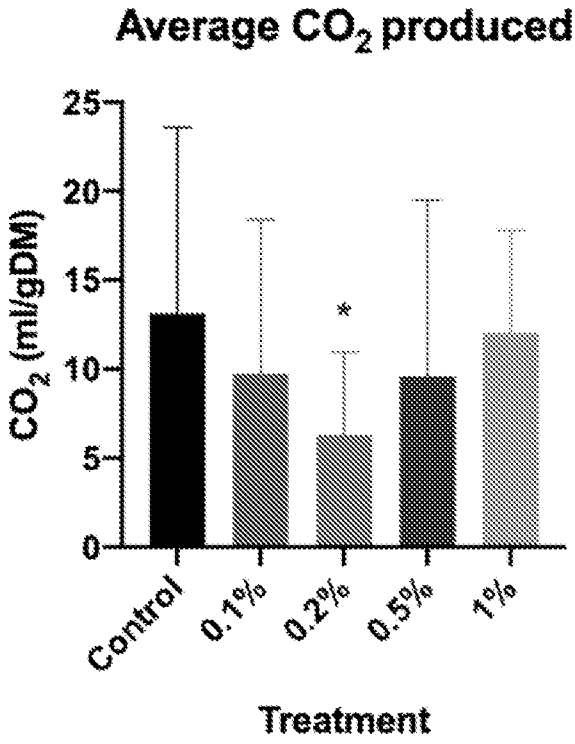
Average CO_2 produced
25
20
15
10
5
0
CO_2 (ml/gDM)
*
Control
0.1%
0.2%
0.5%
1%
Treatment

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/039634

A. CLASSIFICATION OF SUBJECT MATTER

A23K 50/10(2016.01)i; **A23K 10/40**(2016.01)i; **A23K 20/20**(2016.01)i; **A23K 10/30**(2016.01)i; C12R 1/10(2006.01)n; C12R 1/125(2006.01)n

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)

A23K 50/10(2016.01); A01N 25/30(2006.01); A01N 63/00(2006.01); A23K 1/16(2006.01); A23K 1/175(2006.01); A23K 1/18(2006.01); A61K 36/00(2006.01); A61K 36/06(2006.01); A61P 1/00(2006.01); C12N 9/24(2006.01); C12N 9/54(2006.01)

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: digestive health composition, feeding, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis, nutritive mineral component, licking, chewing, livestock animal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2020-076800 A1 (LOCUS IP COMPANY, LLC) 16 April 2020 (2020-04-16) claims 1-35; and pages 2-25	1-9,11-29,31-34
Y		10,30
Y	DASA, KRIS TRIWULAN et al., `Inhibitory effect of long-chain fatty acids on biogas production and the protective effect of membrane bioreactor', BioMed Research International, 08 Sep 2016 (Publication date), Vol. 2016, Article No. 7263974, Internal pages 1-9 internal pages 2, 3	10,30
A	JP 2009-057284 A (KO SAIHATSU) 19 March 2009 (2009-03-19) abstract; and claims 1-13	1-34
A	WO 2020-076797 A1 (LOCUS IP COMPANY, LLC) 16 April 2020 (2020-04-16) abstract; and claims 1-59	1-34

☑ 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
20 October 2021	**21 October 2021**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office **189 Cheongsa-ro, Seo-gu, Daejeon** **35208, Republic of Korea**	**Jung, Da Won**
Facsimile No. **+82-42-481-8578**	Telephone No. **+82-42-481-5373**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012-159186 A1 (GRASP INDÚSTRIA E COMÉRCIO LTDA.) 29 November 2012 (2012-11-29) abstract; and claims 1-9	1-34
A	US 2013-0011384 A1 (MORGAVI, DIEGO P. et al.) 10 January 2013 (2013-01-10) abstract; and claims 14-27	1-34

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2021/039634

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2020-076800	A1	16 April 2020	AU	2019-359209	A1	08 April 2021
				AU	2019-359209	A1	16 April 2020
				BR	112021006787	A2	13 July 2021
				CA	3113996	A1	16 April 2020
				CN	112839523	A	25 May 2021
				EP	3863421	A1	18 August 2021
				KR	10-2021-0057813	A	21 May 2021
JP	2009-057284	A	19 March 2009	WO	2007-072848	A1	28 June 2007
WO	2020-076797	A1	16 April 2020	AU	2019-356502	A1	08 April 2021
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				CN	112839516	A	25 May 2021
				EP	3863408	A1	18 August 2021
				KR	10-2021-0057812	A	21 May 2021
WO	2012-159186	A1	29 November 2012	AR	086529	A1	18 December 2013
				AU	2012-260375	A1	31 October 2013
				AU	2012-260375	A1	29 November 2012
				BR	PI1102284	A2	05 November 2013
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				EP	2713768	A1	09 April 2014
				JP	2014-515265	A	30 June 2014
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				EP	2552231	A1	06 February 2013
				FR	2957754	A1	30 September 2011
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