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(54) Title: PROBIOTIC COMPOSITIONS AND METHODS

(57) Abstract: The present invention relates to probiotic compositions and methods of using such compositions. In particular, the present invention provides methods of using Faecalibacterium spp. and composition derived from culture of Faecalibacterium spp. to prevent or decrease growth of other microorganisms, particularly pathogenic organisms.



## PROBIOTIC COMPOSITIONS AND METHODS

### CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to and the benefit of U.S. Provisional Application No.  
5 62/522,328, filed June 20, 2017, which is hereby incorporated by reference in its entirety.

### FIELD OF INVENTION

The present invention relates to probiotic compositions and methods of using such  
compositions. In particular, the present invention provides methods of using  
10 *Faecalibacterium spp.* and composition derived from culture of *Faecalibacterium spp.* to  
prevent or decrease growth of other microorganisms, particularly pathogenic organisms.

### BACKGROUND OF THE INVENTION

Gut microbiota is known to have a role in shaping key aspects of postnatal life, such  
15 as the development of the immune system (Mazmanian et al., (2005) Cell 122(1): 107-118;  
Peterson et al., (2007) Cell Host Microbe 2(5): 328-339), and influencing the host's  
physiology, including energy balance. Transplanting the gut microbiota from normal mice  
into germ-free recipients increased their body fat without any increase in food consumption,  
raising the possibility that the composition of the microbial community in the gut affects the  
20 amount of energy extracted from the diet (Backhed et al., (2004) Proc Natl Acad Sci U S A  
101(44): 15718-15723). There is at least one type of obesity-associated gut microbiome  
characterised by higher relative abundance of *Firmicutes* or a higher *Firmicutes* to  
*Bacteroidetes* ratio (Ley et al., (2005) Proc Natl Acad Sci U S A 102(31): 11070-11075;  
Turnbaugh et al., (2006) Nature 444(7122): 1027-1031). The role of intestinal microbiota in  
25 disease has also been shown. Gut microbes serve their host by functioning as a key interface  
with the environment; for example, they can protect the host organism from pathogens that  
cause infectious diarrhea. A decreased diversity of fecal microbiota and specifically a  
reduced diversity of *Firmicutes* in Crohn's disease patients has been reported (Manichanh et  
al., (2006) Gut 55(2): 205-211), while it was recently shown that *Faecalibacterium*  
30 *prausnitzii* displays anti-inflammatory action and can potentially be used for the treatment of  
this disease (Sokol et al., (2008) Proc Natl Acad Sci U S A 105(43): 16731-16736).

## SUMMARY OF THE INVENTION

The present invention relates to probiotic compositions and methods of using such compositions. In particular, the present invention provides methods of using *Faecalibacterium spp.* and composition derived from culture of *Faecalibacterium spp.* to prevent or decrease growth of other microorganisms, particularly pathogenic organisms.

Accordingly, in some embodiments, the present invention provides methods of preventing or inhibiting growth of a target microorganism in a subject in need thereof comprising administering to the subject an effective amount of a *Faecalibacterium spp.* composition. In some embodiments, the present invention provides for the use of a *Faecalibacterium spp.* composition to prevent or inhibit growth of a target microorganism. In preferred embodiments, the *Faecalibacterium spp.* strain or strains utilized exhibit or have antimicrobial activity as assayed by an *in vitro* assay.

In some embodiments, the *Faecalibacterium spp.* is *Faecalibacterium prausnitzii*. In some embodiments, the *Faecalibacterium prausnitzii* is strain 24, 30, 266 or 267. In some embodiments, the *Faecalibacterium spp.* composition comprises an effective amount of live *Faecalibacterium spp.* In some embodiments, the *Faecalibacterium spp.* composition comprises an effective amount of dead or inactivated *Faecalibacterium spp.* In some embodiments, the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste. In some embodiments, the *Faecalibacterium spp.* composition further comprises a bulking agent. In some embodiments, the bulking agent is selected from the group consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.

In some embodiments, the *Faecalibacterium spp.* composition is a *Faecalibacterium spp.* culture supernatant from a culture of the *Faecalibacterium spp.* In some embodiments, the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste. In some embodiments, the *Faecalibacterium spp.* composition further comprises a bulking agent. In some embodiments, the bulking agent is selected from the group consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.

In some embodiments, the composition is coadministered with at least a second probiotic organism selected from the group consisting of *Lactobacillus acidophilus*, *L. lactis*, *L. plantarum*, *L. casei*, *Bacillus subtilis*, *B. lichenformis*, *Enterococcus faecium*, *Bifidobacterium bifidum*, *B. longum*, *B. thermophilum*, *Propionibacterium jensenii*, yeast, and combinations thereof.

In some embodiments, the composition is formulated with an additional additive selected from the group consisting of an energy substrate, a mineral, a vitamin, and combinations thereof.

In some embodiments, the target microorganism is a pathogenic microorganism. In some embodiments, the target microorganism is selected from the group consisting of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

In some embodiments, the animal is a domestic animal. In some embodiments, the domestic animal is selected from the group consisting of cattle, sheep, swine, and horses. In some embodiments, the animal is a calf.

In some embodiments, the amount is effective to kill the target microorganism. In some embodiments, the amount is effective to reduce the amount of the microorganism in the organism by at least 50% as compared to the amount of microorganism present prior to administration. In some embodiments, the amount is effective to treat or prevent an infection by the target microorganism.

## DESCRIPTION OF THE FIGURES

**Fig. 1. The effect of *F. prausnitzii* supernatant on the growth of *E. coli* ATCC 31616.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by using the same culture medium. The different proportion of SN (4%, 8%, 16%, 32%, 64%, 100%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by LB broth, respectively. The OD<sub>600</sub> of samples were detected every half an hour until 24 h incubation by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 2. The effect of *F. prausnitzii* supernatant on the growth of *E. coli* ATCC 25922.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by using the same culture medium. The different proportion of SN (8%, 16%, 32%, 64%, 100%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by LB broth, respectively. The OD<sub>600</sub> of samples were detected every half an hour by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 3. The effect of *F. prausnitzii* supernatant on the growth of *Klebsiella pneumoniae* strain 22326.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by

using the same culture medium. The different proportion of SN (8%, 16%, 32%, 64%, 100%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by LB broth, respectively. The OD<sub>600</sub> of samples were detected every half an hour by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 4. The effect of *F. prausnitzii* supernatant on the growth of *Staphylococcus aureus* ATCC 27708.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by using the same culture medium. The different proportion of SN (8%, 16%, 32%, 64%, 100%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by LB broth, respectively. The OD<sub>600</sub> of samples were detected every half an hour by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 5. The effect of *F. prausnitzii* supernatant on the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA) 4454.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by using the same culture medium. The different proportion of SN (8%, 16%, 32%, 64%, 100%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by LB broth, respectively. The OD<sub>600</sub> of samples were detected every half an hour by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 6. The *F. prausnitzii* supernatant cause the reduction of number of *E. coli* ATCC 31616.** The supernatant of *F. prausnitzii* strains were collected after 24 h incubation by using the same culture medium. The cell of *E. coli* ATCC 31616 were recovered after it reached the stationary phase (16 h incubation). The different proportion of SN (4%, 8%, 16%, 32%, 64%) were added into a 96-well plate with a final volume of 300  $\mu$ l supplemented by *E. coli* cells, respectively. The OD<sub>600</sub> of samples were detected every half an hour by using a microwell plate reader (BioTek). The values are expressed as the mean $\pm$ SEM after three independent experiments.

**Fig. 7. CFU counting of different bacteria after co-incubation with the supernatant of *F. prausnitzii* strains 24.** The cell of various bacteria were recovered after it reached the stationary phase. Then the cell of bacteria was co-incubated with a 50% volume of

supernatant of *F. prausnitzii* strains 24. The mixtures of supernatant and *E. coli* were taken at 60 min and serially diluted before CFU counting. The values are expressed as the mean $\pm$ SD after three independent experiments.

5

## DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the term “prokaryotes” refers to a group of organisms that usually  
10 lack a cell nucleus or any other membrane-bound organelles. In some embodiments, prokaryotes are bacteria. The term “prokaryote” includes both archaea and eubacteria.

As used herein, the term “in vitro” refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments can consist of, but are not limited to, test tubes, microtiter plates, and the like. The term “in vivo” refers to  
15 the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within a natural environment.

As used herein, the term “purified” or “to purify” refers to the removal of components (e.g., contaminants) from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of  
20 immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample. In another example, recombinant polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of  
25 recombinant polypeptides is thereby increased in the sample.

As used herein, the term “sample” is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood  
30 products, such as plasma, serum and the like. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

Mammals are defined herein as all animals (e.g., human or non-human animals) that have mammary glands and produce milk.

As used herein, a "dairy animal" refers to a milk producing non-human mammal that is larger than a laboratory rodent (*e.g.*, a mouse). In preferred embodiments, the dairy animals produce large volumes of milk and have long lactating periods (*e.g.*, cows or goats).

A "subject" is an animal such as vertebrate, preferably a domestic animal or a  
5 mammal. Mammals are understood to include, but are not limited to, murines, simians, humans, bovines, cervids, equines, porcines, canines, felines *etc.*

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations,

"Co-administration" refers to administration of more than one agent or therapy to a  
10 subject. Co-administration may be concurrent or, alternatively, the chemical compounds described herein may be administered in advance of or following the administration of the other agent(s). One skilled in the art can readily determine the appropriate dosage for co-administration. When co-administered with another therapeutic agent, both the agents may be used at lower dosages. Thus, co-administration is especially desirable where the claimed  
15 compounds are used to lower the requisite dosage of known toxic agents.

As used herein, the term "toxic" refers to any detrimental or harmful effects on a cell or tissue.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or  
20 therapeutic use *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and an emulsion, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers,  
25 stabilizers and adjuvants see Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, PA (1975).

"Pharmaceutically acceptable salt" as used herein, relates to any pharmaceutically acceptable salt (acid or base) of a compound of the present invention, which, upon administration to a recipient, is capable of providing a compound of this invention or an  
30 active metabolite or residue thereof. As is known to those of skill in the art, "salts" of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-

sulfonic and benzenesulfonic acid. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid.

5 As used herein, the term "nutraceutical," refers to a food substance or part of a food, which includes a probiotic bacterium. Nutraceuticals can provide medical or health benefits, including the prevention, treatment, or cure of a disorder.

The terms "bacteria" and "bacterium" refer to all prokaryotic organisms, including those within all of the phyla in the Kingdom Procaryotae. It is intended that the term  
10 encompass all microorganisms considered to be bacteria including Mycoplasma, Chlamydia, Actinomyces, Streptomyces, and Rickettsia. All forms of bacteria are included within this definition including cocci, bacilli, spirochetes, spheroplasts, protoplasts, *etc.* Also included within this term are prokaryotic organisms that are gram negative or gram positive. "Gram negative" and "gram positive" refer to staining patterns with the Gram-staining process that is  
15 well known in the art. (*See e.g.*, Finegold and Martin, Diagnostic Microbiology, 6th Ed., CV Mosby St. Louis, *pp.* 13-15 [1982]). "Gram positive bacteria" are bacteria that retain the primary dye used in the Gram stain, causing the stained cells to appear dark blue to purple under the microscope. "Gram negative bacteria" do not retain the primary dye used in the Gram stain, but are stained by the counterstain. Thus, gram negative bacteria appear red.

20

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to probiotic compositions and methods of using such compositions. In particular, the present invention provides methods of using  
*Faecalibacterium spp.* and composition derived from culture of *Faecalibacterium spp.* to  
25 prevent or decrease growth of other microorganisms, particularly pathogenic organisms.

Changes in the microbial community are observed in individuals with intestinal inflammatory disorders. These changes are often accompanied by a decrease of obligate anaerobic bacteria, whereas the relative abundance of facultative anaerobic Enterobacteriaceae increases (Winter, et al., 2013). Inflammatory bowel diseases (IBD),  
30 including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial ailments characterized by intestinal inflammation (Huang, et al., 2016). *Faecalibacterium prausnitzii*, is an extremely oxygen sensitive commensal butyrate producer bacterium belonging to the *Clostridium leptum* group of the gut microbiota, recognized as a biomarker of intestinal health (Miquel, et al., 2013). *F. prausnitzii* is an anti-inflammatory commensal bacterium

identified on the basis of human clinical data (Hornef, et al., 2016). *F. prausnitzii* produces high amounts of butyrate, which has well known anti-inflammatory effects (Segain, et al., 2000). A previous study (Winter, et al., 2013) showed that nitrate generated as a by-product of the inflammatory response conferred a growth advantage to the commensal bacterium *E. coli* in the large intestine of mice. However, the mechanisms underlying its beneficial effects on human and animal health are still unknown.

The reduction of *F. prausnitzii* was associated with higher risk postoperative recurrence of ileal Crohn Disease (CD) (Sokol, et al., 2008). A significantly lower proportion of *F. prausnitzii* at the time of surgery, consistently associated with endoscopic relapse was observed. In vitro peripheral blood mononuclear cell stimulation by *F. prausnitzii* led to significantly lower IL-12 and IFN- $\gamma$  production levels and higher secretion of IL-10. Furthermore, oral administration of either live *F. prausnitzii* or its supernatant markedly reduced the severity of TNBS colitis and tended to correct the dysbiosis associated with TNBS colitis (Sokol, et al., 2008). Moreover, *F. prausnitzii* was suggested to induce a similar interleukin 10 (IL-10)-producing regulatory T cell population in humans (Sarrabayrouse, et al., 2014). The FP A2-165 reference strain significantly decreased colonic hypersensitivity induced by either NMS in mice or partial restraint stress in rats (Miquel, et al., 2016). Faecalibacterium prausnitzii is strongly associated with Atopic Dermatitis (AD). A recent study showed that the fecal samples from patients with AD showed decreased levels of butyrate and propionate, which have anti-inflammatory effects (Song, et al., 2016).

*F. prausnitzii* may also be involved in reduction of colitis through in vivo modulation of metabolites. Previous studies demonstrated that metabolites of *F. prausnitzii* could affect gut physiology and immunity. The supernatant from *F. prausnitzii* cultures significantly reduced IL-8 secretion induced by IL-1 $\beta$  (Sokol, et al., 2008). Interestingly, Sokol, et al., 2008 also examined the antimicrobial effects of *F. prausnitzii* supernatant and found no such effect *in vitro*. Moreover, salicylic acid was found directly involved in the protective effect of *F. prausnitzii* (Miquel, et al., 2015). Additionally, after treatment with *F. prausnitzii* supernatant, the plasma levels of IL-17A and IL-6 ( $P<0.05$ ), the protein and mRNA expression of IL-17A and ROR $\gamma$ t, and the Th17 cell ratio of spleen cells ( $P<0.01$ ) were significantly decreased compared to the model group (Huang, et al., 2016).

A recent study demonstrated that a peptide, identified in the *F. prausnitzii* culture supernatants, has an anti-inflammatory effect (Quevrain, et al., 2016). Two fractions (F2 and F3) from *F. prausnitzii* supernatant exerted inhibitory effects on IL1- $\beta$ -induced IL-8 secretion in intestinal epithelial CaCO2 cells. Interestingly, the seven peptides derived from a single

microbial anti-inflammatory molecule (MAM), a protein of 15 kDa, and comprising 53% of non-polar residues. Transfection of MAM cDNA in epithelial cells led to a significant decrease in the activation of the nuclear factor (NF)- $\kappa$ B pathway with a dose-dependent effect (Quevrain, et al., 2016). They also observed that anti-inflammatory effect was not abrogated by heat (above 70°C), enzyme digestion (trypsin, lipase, amylase) or MW filtration acid and HBTU/DIEA for the activation. The crude peptides were purified by HPLC to obtain purity over 97% (Quevrain, et al., 2016). These results indicated that various metabolites other than MAM could contribute to this effect.

Short-chain fatty acids (SCFA) such as acetate, n-propionate, n-butyrate are end products of bacterial anaerobic fermentation of dietary fiber and are likely candidates for regulating immune response in the intestines (Chang, et al., 2014). SCFA can be found at high concentrations in the large intestine (e.g., 20 mM n-butyrate in colonic lumen) (Louis, et al., 2009). Chang et al demonstrated that the n-butyrate can modulate the function of intestinal macrophages. Treatment of macrophages with n-butyrate led to the down-regulation of lipopolysaccharide-induced proinflammatory mediators, including nitric oxide, IL-6 and IL-12, but did not affect levels of TNF- $\alpha$  or MCP-1 (Chang, et al., 2014).

The anti-inflammatory effect exerted by *F. prausnitzii* was associated with various metabolic changes but the precise molecular mechanisms remained undefined. Targeting its metabolic pathways could be an attractive therapeutic strategy in IBD (Miquel, et al., 2015).

While these anti-inflammatory effects have been described, there has been no previous disclosure that *F. prausnitzii* compositions have antibacterial activity. Experiments described herein demonstrate that *F. prausnitzii* compositions have antibacterial activity, and for example, inhibit the growth of or kill bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Accordingly, the present invention provides methods of using *Faecalibacterium spp.* and compositions derived from cultures of *Faecalibacterium spp.* to prevent or decrease growth of other microorganisms, particularly pathogenic organisms. The *F. prausnitzii* compositions of the present invention may comprise live or dead strain(s) of *F. prausnitzii* or may be *F. prausnitzii* supernatants obtained from culturing *F. prausnitzii*. In some preferred embodiments, the *F. prausnitzii* used in the compositions and methods of the present invention is isolated as described in Foditsch et al., PLOS ONE | DOI:10.1371/journal.pone.0116465(2014), incorporated herein by reference in its entirety. It will be understood to those of ordinary skill in the art that suitable strains of *F. prausnitzii* may be obtained as described in Foditsch (2014) and screened as described herein for

antimicrobial activity. Thus, the current invention is not limited to the specific strains described herein. Multiple strains with antimicrobial activity are described in the examples and additional strains for use in the methods and compositions of the present invention can be obtained as described herein.

5           In some embodiments, compositions and formulations of the present invention comprise an effective amount of a *F. prausnitzii* composition, for example, one or more live or dead *F. prausnitzii* strains (or combinations thereof) or a *F. prausnitzii* culture supernatant. In some embodiments, the effective amount is an amount sufficient to inhibit the growth of a target microorganism. In some embodiments, the effective amount is an amount sufficient to  
10       prevent the growth of a target microorganism. In some embodiments, the effective amount is an amount sufficient to kill the target microorganism. In some embodiments, the amount is effective to reduce the amount of the microorganism in the subject by at least 99%, 90%, 80%, 70%, 60% or 50% as compared to the amount of microorganism present prior to administration. The amount of the microorganism present can be determined, for example,  
15       by culturing a sample or swab taken from a subject that is being treated. In some embodiments, the amount is effective to treat or prevent an infection by the target microorganism.

### **I. Compositions and Kits**

20           In some embodiments, the present invention provides *F. prausnitzii* compositions and kits. In some embodiments, *F. prausnitzii* compositions comprise one or more live and/or dead *Faecalibacterium spp.* or strains. The present invention is not limited to a particular *Faecalibacterium spp.* or strain. Examples include, but are not limited to, *Faecalibacterium prausnitzii*, and in particularly preferred embodiments include strains 24, 30, 266 and 267.

25           In some embodiments, compositions comprise one or more additional components (e.g., including but not limited to, additional additive selected from the group consisting of an energy substrate, a mineral, a vitamin, or combinations thereof).

          In some embodiments, compositions comprise one or more (e.g., 2 or more, 5 or more, 10 or more, etc.) additional strains of bacteria or other microorganisms (e.g., probiotic  
30       microorganisms). Examples include, but are not limited to, *Lactobacillus acidophilus*, *L. lactis*, *L. plantarum*, *L. casei*, *Bacillus subtilis*, *B. licheniformis*, *Enterococcus faecium*, *Bifidobacterium bifidum*, *B. longum*, *B. thermophilum*, *Propionibacterium jensenii*, yeast, or combinations thereof. In some embodiments, multiple strains of the same bacteria are utilized in combination.

In some embodiments, bacteria are live cells or freeze-dried cells. Freeze-dried bacteria can be stored for several years with maintained viability. In certain applications, freeze-dried bacteria are sensitive to humidity. One way of protecting the bacterial cells is to store them in oil. The freeze dried bacterial cells can be mixed directly with a suitable oil, or  
5 alternately the bacterial cell solution can be mixed with an oil and freeze-dried together, leaving the bacterial cells completely immersed in oil. Suitable oils may be edible oils such as olive oil, rapeseed oil which is prepared conventionally or cold-pressed, sunflower oil, soy oil, maize oil, cotton-seed oil, peanut oil, sesame oil, cereal germ oil such as wheat germ oil, grape kernel oil, palm oil and palm kernel oil, linseed oil. The viability of freeze-dried  
10 bacteria in oil is maintained for at least nine months. Optionally live cells can be added to one of the above oils and stored.

In some embodiments, the *F. prausnitzii* compositions are supernatants from a culture of *F. prausnitzii*. The supernatants resulting from the culture of *F. prausnitzii* may preferably be dried, for example by spray drying, vacuum drying, freeze-drying or lyophilization. The  
15 resulting powder may preferably be formulated as a powder, such as a dispersible powder, bolus, gel, liquid drench, capsule, or paste

In some embodiments, the *F. prausnitzii* compositions are part of a milk replacer (e.g., for administration to a neonatal or young animal). In some embodiments, compositions comprise one or more probiotic bacteria as described herein in combination with a milk  
20 protein (e.g., caseins or whey proteins).

In some embodiments, *F. prausnitzii* compositions are added to nutraceuticals, food products, or foods. In some embodiments, to give the composition or nutraceutical a pleasant taste, flavoring substances such as for example mints, fruit juices, licorice, Stevia rebaudiana, steviosides or other calorie free sweeteners, rebaudioside A, essential oils like eucalyptus oil,  
25 or menthol can optionally be included in compositions of embodiments of the present invention.

In some embodiments, *F. prausnitzii* compositions are formulated in pharmaceutical compositions. The bacteria of embodiments of the invention may be administered alone or in combination with pharmaceutically acceptable carriers or diluents, and such administration  
30 may be carried out in single or multiple doses.

Compositions may, for example, be in the form of tablets, resolvable tablets, capsules, bolus, drench, pastes, pills sachets, vials, hard or soft capsules, aqueous or oily suspensions, aqueous or oily solutions, emulsions, powders, granules, syrups, elixirs, lozenges, reconstitutable powders, liquid preparations, creams, troches, hard candies, sprays, chewing-

gums, creams, salves, jellies, gels, pastes, toothpastes, rinses, dental floss and tooth-picks, liquid aerosols, dry powder formulations, HFA aerosols or organic or inorganic acid addition salts.

The pharmaceutical compositions of embodiments of the invention may be in a form suitable for oral, topical, buccal administration. Depending upon the disorder and subject to be treated and the route of administration, the compositions may be administered at varying doses.

For oral or buccal administration, bacteria of embodiments of the present invention may be combined with various excipients. Solid pharmaceutical preparations for oral administration often include binding agents (for example syrups, acacia, gelatin, tragacanth, polyvinylpyrrolidone, sodium lauryl sulphate, pregelatinized maize starch, hydroxypropyl methylcellulose, starches, modified starches, gum acacia, gum tragacanth, guar gum, pectin, wax binders, microcrystalline cellulose, methylcellulose, carboxymethylcellulose, hydroxypropyl methylcellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, copolyvidone and sodium alginate), disintegrants (such as starch and preferably corn, potato or tapioca starch, alginic acid and certain complex silicates, polyvinylpyrrolidone, gelatin, acacia, sodium starch glycollate, microcrystalline cellulose, crosscarmellose sodium, crospovidone, hydroxypropyl methylcellulose and hydroxypropyl cellulose), lubricating agents (such as magnesium stearate, sodium lauryl sulfate, talc, silica polyethylene glycol waxes, stearic acid, palmitic acid, calcium stearate, carnuba wax, hydrogenated vegetable oils, mineral oils, polyethylene glycols and sodium stearyl fumarate) and fillers (including high molecular weight polyethylene glycols, lactose, calcium phosphate, glycine magnesium stearate, starch, rice flour, chalk, gelatin, microcrystalline cellulose, calcium sulphate, and lactitol). Such preparations may also include preservative agents and anti-oxidants.

Liquid compositions for oral administration may be in the form of, for example, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid compositions may contain conventional additives such as suspending agents (e.g. syrup, methyl cellulose, hydrogenated edible fats, gelatin, hydroxyalkylcelluloses, carboxymethylcellulose, aluminium stearate gel, hydrogenated edible fats) emulsifying agents (e.g. lecithin, sorbitan monooleate, or acacia), aqueous or non-aqueous vehicles (including edible oils, e.g. almond oil, fractionated coconut oil) oily esters (for example esters of glycerine, propylene glycol, polyethylene glycol or ethyl alcohol), glycerine, water or normal saline; preservatives (e.g. methyl or propyl p-hydroxybenzoate or sorbic acid) and conventional flavoring, preservative, sweetening or

coloring agents. Diluents such as water, ethanol, propylene glycol, glycerin and combinations thereof may also be included.

Other suitable fillers, binders, disintegrants, lubricants and additional excipients are well known to a person skilled in the art.

5           In some embodiments, bacteria are spray-dried. In other embodiments, bacteria are suspended in an oil phase and are encased by at least one protective layer, which is water-soluble (water-soluble derivatives of cellulose or starch, gums or pectins; See e.g., EP 0 180 743, herein incorporated by reference in its entirety).

10           In some embodiments, the present invention provides kits, pharmaceutical compositions, or other delivery systems for use in preventing or inhibiting growth of a target microorganism in a subject. The kit may include any and all components necessary, useful or sufficient for research or therapeutic uses including, but not limited to, one or more probiotic bacteria or supernatant compositions, pharmaceutical carriers, and additional components useful, necessary or sufficient for improving weight gain, providing prophylaxis against  
15           diarrhea and/or improving feed efficiency in an animal. In some embodiments, the kits provide a sub-set of the required components, wherein it is expected that the user will supply the remaining components. In some embodiments, the kits comprise two or more separate containers wherein each container houses a subset of the components to be delivered.

20           Optionally, compositions and kits comprise other active components in order to achieve desired therapeutic effects.

## II.     Therapeutic and Supplement Uses

Embodiments of the present invention provide *Faecalibacterium spp.* compositions (e.g., *F. prausnitzii* compositions as described above alone or in combination with additional  
25           probiotic bacteria) for use in inhibiting or preventing growth of a microorganism in a subject, or for killing a microorganism in a subject, or for treating or preventing an infection by a microorganism in a subject. In preferred embodiments, an effective amount of the *F. prausnitzii* composition or formulation is administered to the subject under conditions that growth of a target microorganism such as *Escherichia coli*, *Klebsiella pneumoniae* or  
30           *Staphylococcus aureus* is prevented or inhibited, or so that the microorganism is killed.

## EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

5 **Example 1**

The *F. prausnitzii* strains used in the following examples were isolated as described in Foditsch et al., PLOS ONE | DOI:10.1371/journal.pone.0116465(2014). Briefly, fecal samples were collected from 7–28 days-old healthy Holstein calves and from 10–30 days-old healthy piglets. The samples were gently collected from the rectum and immediately placed in a tube containing 12 ml of VTR2RF broth. The tubes were sealed and transported until further processing. The subsequent procedures were performed in an anaerobic chamber (BacBasic chamber, Sheldon Manufacturing, Inc., Cornelius, OR). All samples were serially diluted in Anaerobic Dilution Blank (Anaerobe Systems, USA) and plated on VTR2RF agar. After 48 h, about 10 typical colonies from each sample were selected and single- colony purified in VTR2RF agar. The isolates were stored at 280°C in VTR2RF broth containing 16% of glycerol.

The VTR2RF media used as transport, enrichment, and isolation media was composed of the anaerobic media Versa TREK REDOX 2 (Trek Diagnostic Systems, Cleveland-OH) supplemented with 30% filtered rumen fluid. Rumen fluid was collected from fistulated cows, centrifuged at 12,000g for 30 min, the supernatant was filter-sterilized (Corning Incorporated Life Sciences, Tewksbury-MA) 3 times and stored at 4°C. VTR2RF agar was additionally supplemented with 0.5% (w/v) yeast extract (BD, Franklin Lakes, NJ), 5 mg/l (w/v) hemin (Sigma-Aldrich, St. Louis, MO), 1 mg/l (w/v) cellobiose (Sigma-Aldrich), 1 mg/ml (w/v) maltose (Sigma-Aldrich), and 0.5 mg/ml (w/v) L-cystein (Sigma-Aldrich).

### Example 2

### 1. Screening of significant *F. prausnitzii* strains

30 To investigate if *F. prausnitzii* supernatant (SN) could inhibit the growth of *E. coli*, a batch of *F. prausnitzii* strains were selected to carry out the growth inhibition test, which included 15 strains (strain 4, 24, 24D, 27, 30, 32, 34, 36D, 58, 69D, 74, 266, 267, 272X and 297X). *F. prausnitzii* A2-165 (DSM 17677) was used as a control strain. The enterotoxigenic

*Escherichia coli* ATCC 31616 (K19), known to induce diarrhea in animals, was used as the *E. coli* treatment strain.

Results showed that the supernatant of *F. prausnitzii* strains 24, 30, 266 and 267 displayed a significant inhibition effect on the growth of *Escherichia coli* ATCC 31616 (Figure 1). There was no significant effect by adding the supernatant of *F. prausnitzii* DSM 17677 or the medium for the growth of *F. prausnitzii* strains. Moreover, the inhibition effect increased with increases in the percentage of *F. prausnitzii* supernatant, which indicates that the inhibition effect is dose-dependent. Interestingly, the growth of *E. coli* ATCC 31616 was completely suppressed by adding 64% or 100% supernatant of *F. prausnitzii* strain 266 and strain 267 into the LB broth for the growth of *E. coli* ATCC 31616. Additionally, for the supernatant of *F. prausnitzii* strain 24, it caused a significant decrease in the number of *E. coli* ATCC 31616 after the stationary phase, which indicates that the supernatant of *F. prausnitzii* strain 24 kills *E. coli* ATCC 31616.

The supernatant of *F. prausnitzii* strains 24, 30, 266 and 267 had significant inhibition effect on the growth of *E. coli* ATCC 31616, and was selected for further study.

## 2. The supernatant of *F. prausnitzii* displayed widespread inhibition effect on *E. coli*

In order to explore if the inhibition effect of *F. prausnitzii* supernatant is specific to *E. coli* ATCC 31616, we investigated the effect of the supernatant of *F. prausnitzii* strains 24, 30, 266 and 267 on the growth of *E. coli* ATCC 25922, a non-pathogenic *E. coli* reference strain.

Significant inhibition on the growth of *E. coli* ATCC 25922 was observed, which displayed a dose-dependent manner (Figure 2). In the same way, the growth of *E. coli* ATCC 25922 was completely suppressed by adding >32% supernatant of *F. prausnitzii* strains into the LB broth for the growth of *E. coli*. These results show that the inhibition effect of *F. prausnitzii* supernatant is universal for various *E. coli* strains.

## 3. The supernatant of *F. prausnitzii* displayed significant inhibition effect on *Klebsiella pneumoniae*

In order to explore if the inhibition effect of *F. prausnitzii* supernatant is specific to *E. coli*, we carried out the growth inhibition test on *Klebsiella pneumoniae* strain 22326, a pathogenic *Klebsiella pneumoniae* strain that induces mastitis in dairy cows. Results showed that the supernatant of *F. prausnitzii* strain 24, 30, 266 and 267 has significant inhibition effects on the growth of *Klebsiella pneumoniae* strain 22326 in a dose-dependent manner

(Figure 3). Similarly, the OD<sub>600</sub> of *Klebsiella pneumoniae* decreased significantly by adding the supernatant of *F. prausnitzii* strain 24 after 18 h incubation.

#### 4. The supernatant of *F. prausnitzii* displayed significant inhibition effect on *Staphylococcus aureus*

In order to explore if the inhibition effect of *F. prausnitzii* supernatant is specific to Gram negative bacteria, growth inhibition studies were carried out using *Staphylococcus aureus* ATCC 27708. Results showed that the supernatant from all four strains of *F. prausnitzii* had significant inhibition effects on the growth of *Staphylococcus aureus* ATCC 27708 in a dose-dependent manner. Similarly, the OD<sub>600</sub> of *Staphylococcus aureus* ATCC 27708 decreased significantly by adding the supernatant of *F. prausnitzii* strain 24 after 18 h incubation (Figure 4).

#### 5. The supernatant of *F. prausnitzii* displayed significant inhibition effect on Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is a serious type of *S. aureus* bacteria that is resistant to many different kinds of antibiotics. In order to explore if the inhibition effect of *F. prausnitzii* supernatant is applicative to MRSA, MRSA 4454 was selected to do the growth inhibition tests. Results showed that supernatants from *F. prausnitzii* strains 24, 30, 266 and 267 have significant inhibition effect on the growth of MRSA in a dose-dependent manner. Similarly, the OD<sub>600</sub> of MRSA cell decreased significantly by adding the supernatant of *F. prausnitzii* strain 24 after 18 h incubation (Figure 5).

### Example 3

#### 1. The supernatant of *F. prausnitzii* strain 24 kills *E. coli* ATCC 31616

In our previous study, we demonstrated that the number of *E. coli* ATCC 31616 (K19) decreased after 16 h incubation with the supernatant of *F. prausnitzii* strain 24. Then we further explored if the supernatant of *F. prausnitzii* strain 24 could kill *E. coli* ATCC 31616.

*E. coli* ATCC 31616 was recovered after reaching the stationary phase. The OD<sub>600</sub> of culture by adding *F. prausnitzii* supernatant into the cell were detected every half an hour until 8 h. Results showed that the OD<sub>600</sub> of culture decreased significantly for the group of *F. prausnitzii* strain 24 (Figure 6). This result agreed with the previous study that the number of *E. coli* ATCC 31616 reduced after 16 h incubation with the supernatant of *F. prausnitzii* strain 24.

## 2. The supernatant of *F. prausnitzii* strain 24 significantly decreased the number of different bacteria

To verify our hypothesis, we further carried out CFU counting tests after the co-incubation of various bacteria with the supernatant of *F. prausnitzii* strain 24. A total of 4 different kinds of bacteria were selected to do this experiment, including a common non-pathogenic *E. coli* reference strain *E. coli* ATCC 25922, two important pathogenic *E. coli* reference strains *E. coli* ATCC 31616 (K19) and *E. coli* ATCC #55, and a pathogenic *Klebsiella pneumoniae* that induces mastitis in dairy cows, *K. pneumoniae* strain 22326. The cell of various bacteria were recovered after reaching the stationary phase. Then the cell of bacteria was co-incubated with the supernatant of *F. prausnitzii* strain 24. The mixtures of supernatant and *E. coli* were taken at 60 min and serially diluted. These dilutions were plated on LB agar and incubated overnight to determine cell viability and number of CFU. The results showed that the supernatant of *F. prausnitzii* strain 24 maintained its inhibitory activity to all the test strains (**Figure 7**). Compared with the control group, supernatant of *F. prausnitzii* strain DSM 17677 and culture medium, a progressive reduction in bacterial population was observed after co-incubation with the supernatant of *F. prausnitzii* strain 24.

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5           All publications, patents, patent applications and accession numbers mentioned in the  
above specification are herein incorporated by reference in their entirety. Although the  
invention has been described in connection with specific embodiments, it should be  
understood that the invention as claimed should not be unduly limited to such specific  
embodiments. Indeed, various modifications and variations of the described compositions  
10   and methods of the invention will be apparent to those of ordinary skill in the art and are  
intended to be within the scope of the following claims.

**CLAIMS**

1. A method of preventing or inhibiting growth of a target microorganism in a subject in need thereof comprising administering to the subject an effective amount of a  
5 *Faecalibacterium spp.* composition.
2. The method of claim 1, wherein the *Faecalibacterium spp.* is *Faecalibacterium prausnitzii*.
- 10 3. The method of claim 1 or 2, wherein said *Faecalibacterium spp.* has antimicrobial activity.
4. The method of claim 3, wherein said effective amount of *Faecalibacterium spp.* prevents, inhibits the growth of, or kills a bacteria selected from the group consisting of  
15 *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and combinations thereof.
5. The method of claim 4, wherein said *Escherichia coli* is *Escherichia coli* ATCC 31616 (K19).
- 20 6. The method of any one of claims 1 to 5, wherein the *Faecalibacterium spp.* composition comprises an effective amount of dead or inactivated *Faecalibacterium spp.*
7. The method of any one claims 5 to 6, wherein the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste.  
25
8. The method of claim 7, wherein the *Faecalibacterium spp.* composition further comprises a bulking agent.
9. The method of claim 8, wherein the bulking agent is selected form the group  
30 consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.
10. The method of any one of claims 1 to 5, wherein the *Faecalibacterium spp.* composition is a *Faecalibacterium spp.* culture supernatant from a culture of the *Faecalibacterium spp.*

11. The method of claim 10, wherein the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste.

5 12. The method of claim 11, wherein the *Faecalibacterium spp.* composition further comprises a bulking agent.

13. The method of claim 12, wherein the bulking agent is selected form the group consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.

10

14. The method of any one of claims 1 to 13, wherein the composition is coadministered with at least a second probiotic organism selected from the group consisting of *Lactobacillus acidophilus*, *L. lactis*, *L. plantarum*, *L. casei*, *Bacillus subtilis*, *B. lichenformis*, *Enterococcus faecium*, *Bifidobacterium bifidum*, *B. longum*, *B. thermophilum*, *Propionibacterium jensenii*,  
15 yeast, and combinations thereof.

15. The method any one of claims 1 to 14, wherein the composition is formulated with an additional additive selected from the group consisting of an energy substrate, a mineral, a vitamin, and combinations thereof.

20

16. The method of any one of claims 1 to 15, wherein the target microorganism is a pathogenic microorganism.

17. The method of any one of claims 1 to 16, wherein the target microorganism is  
25 selected from the group consisting of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

18. The method of any one of claims 1 to 17, wherein the subject is a domestic animal.

30 19. The method of claim 18, wherein the domestic animal is selected from the group consisting of cattle, sheep, swine, and horses.

20. The method of claim 19, wherein the animal is a calf.

21. The method of any one of claims 1 to 20, wherein the amount is effective to kill the target microorganism.

22. The method of any one of claims 1 to 21, wherein the amount is effective to reduce  
5 the amount of the microorganism in the organism by at least 50% as compared to the amount of microorganism present prior to administration.

23. Use of a *Faecalibacterium spp.* composition having antimicrobial activity to prevent or inhibit growth of or kill a target microorganism.

10

24. Use of claim 22, wherein the *Faecalibacterium spp.* is *Faecalibacterium prausnitzii*.

25. Use of claim 23 or 24, wherein said *Faecalibacterium spp.* has antimicrobial activity.

15

26. Use of claim 25, wherein said effective amount of *Faecalibacterium spp.* prevents, inhibits the growth of, or kills a bacteria selected from the group consisting of *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and combinations thereof.

20

27. The method of claim 26, wherein said *Escherichia coli* is *Escherichia coli* ATCC 31616 (K19).

28. Use of any one of claims 23 to 27, wherein the *Faecalibacterium spp.* composition comprises live *Faecalibacterium spp.*

25

29. Use of any one of claims 25 to 27, wherein the *Faecalibacterium spp.* composition comprises dead or inactivated *Faecalibacterium spp.*

30. Use of any one claims 28 to 29, wherein the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste.

30

31. Use of claim 30, wherein the *Faecalibacterium spp.* composition further comprises a bulking agent.

32. Use of claim 31, wherein the bulking agent is selected from the group consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.

33. Use of any one of claims 23 to 27, wherein the *Faecalibacterium spp.* composition is  
5 a *Faecalibacterium spp.* culture supernatant from a culture of the *Faecalibacterium spp.*

34. Use of claim 33, wherein the *Faecalibacterium spp.* composition is formulated powder, bolus, gel, liquid drench, capsule, or paste.

10 35. Use of claim 34, wherein the *Faecalibacterium spp.* composition further comprises a bulking agent.

36. Use of claim 35, wherein the bulking agent is selected from the group consisting of milk powder, skim milk powder, corn starch, corn meal, and soybean meal.

15

37. Use of any one of claims 23 to 36, wherein the composition is coadministered with at least a second probiotic organism selected from the group consisting of *Lactobacillus acidophilus*, *L. lactis*, *L. plantarum*, *L. casei*, *Bacillus subtilis*, *B. lichenformis*, *Enterococcus faecium*, *Bifidobacterium bifidum*, *B. longum*, *B. thermophilum*, *Propionibacterium jensenii*,  
20 yeast, and combinations thereof.

38. Use any one of claims 23 to 37, wherein the composition is formulated with an additional additive selected from the group consisting of an energy substrate, a mineral, a vitamin, and combinations thereof.

25

39. Use of any one of claims 23 to 38, wherein the target microorganism is a pathogenic microorganism.

40. Use of any one of claims 23 to 39, wherein the target microorganism is selected from  
30 the group consisting of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

41. Use of any one of claims 23 to 34, wherein the subject is a domestic animal.

42. Use of claim 41, wherein the domestic animal is selected from the group consisting of cattle, sheep, swine, and horses.

43. Use of claim 42, wherein the animal is a calf.

5

44. Use of any one of claims 23 to 42, wherein the *Faecalibacterium spp.* composition is provided in an effective amount.

45. Use of claim 44 wherein the amount is effective to kill the target microorganism.

10

46. Use of claim 44, wherein the amount is effective to reduce the amount of the microorganism in the organism by at least 50% as compared to the amount of microorganism present prior to administration.

15

47. Use of claim 44, wherein the amount is effective to treat or prevent an infection by the target microorganism.

FIG. 1

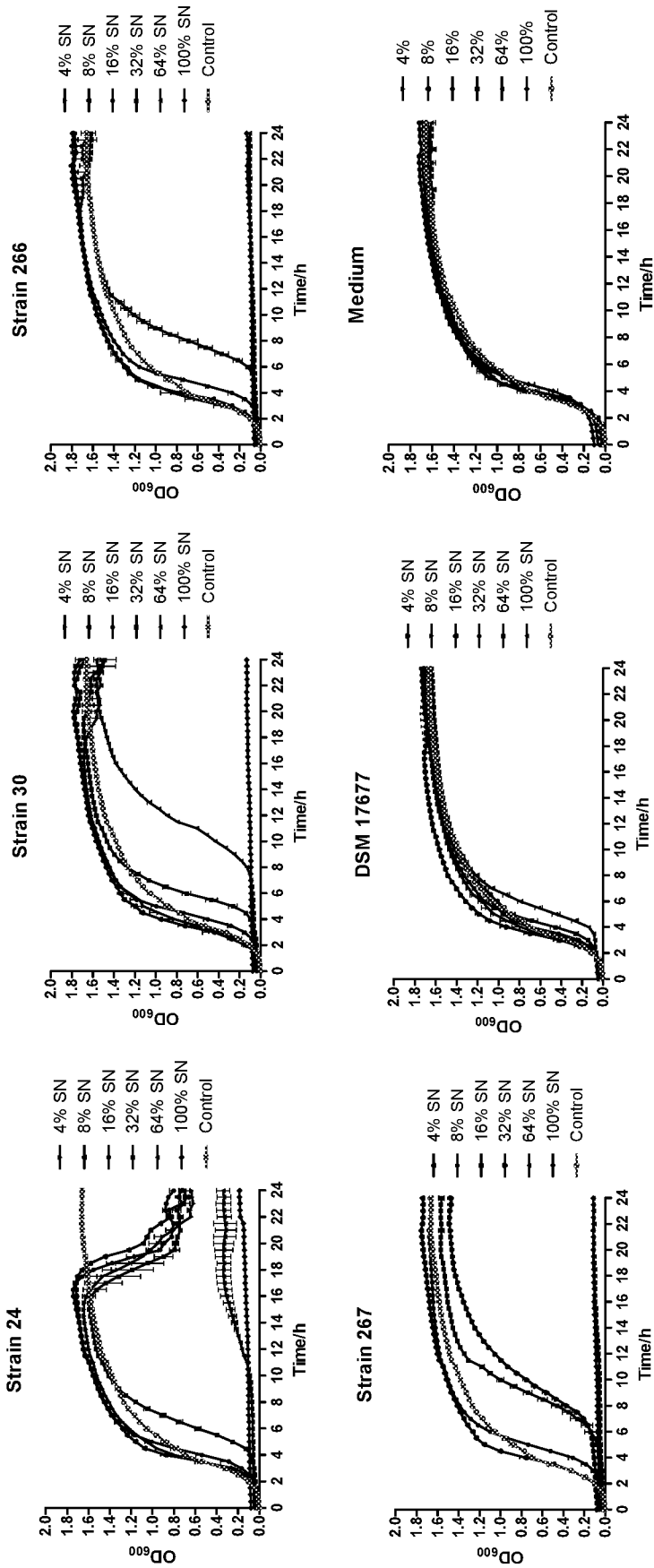


FIG. 2

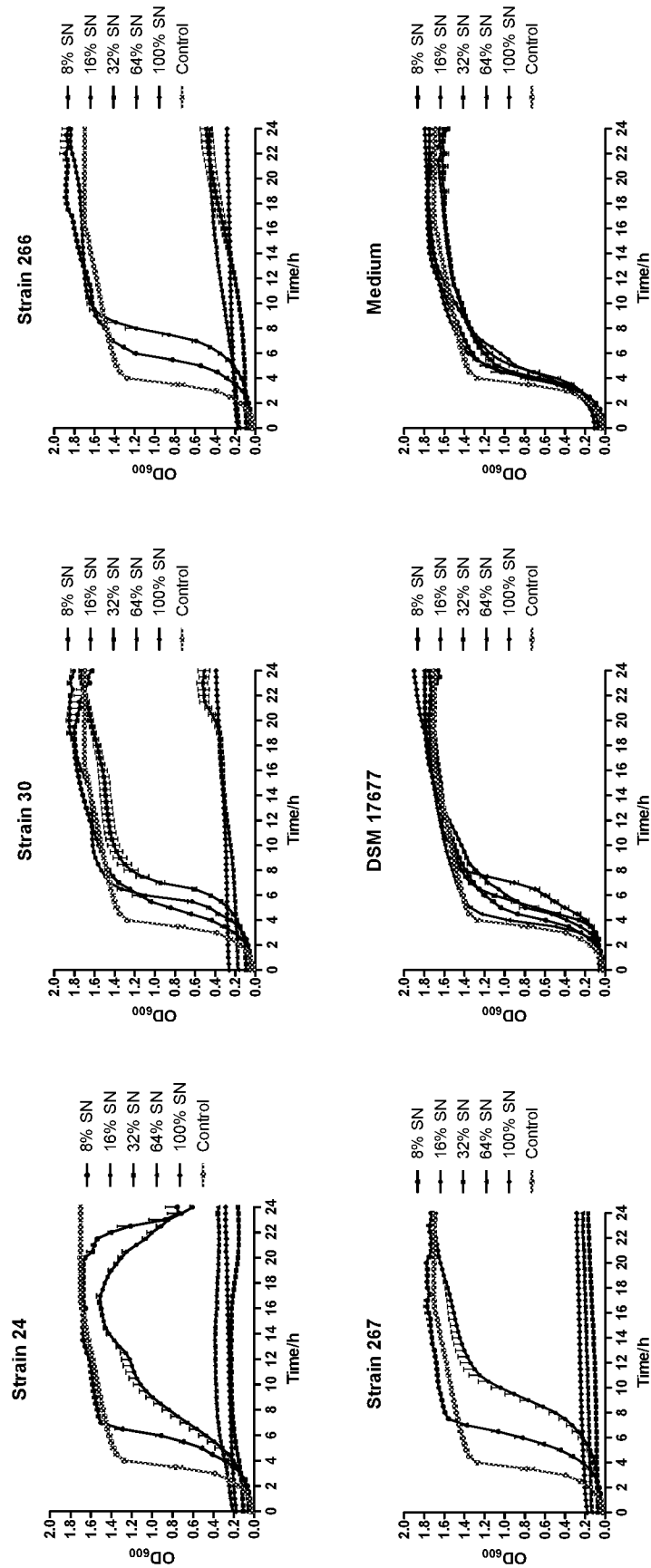


FIG. 3

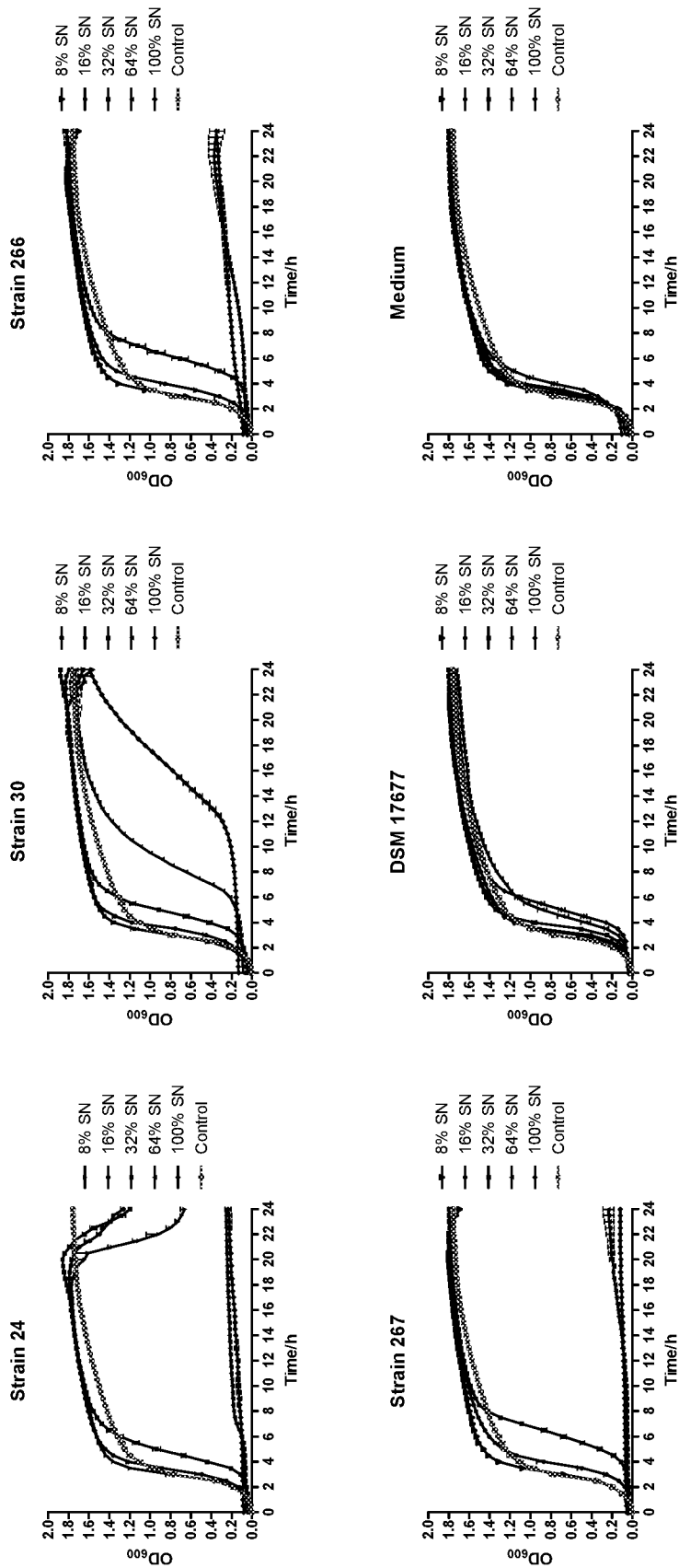


FIG. 4

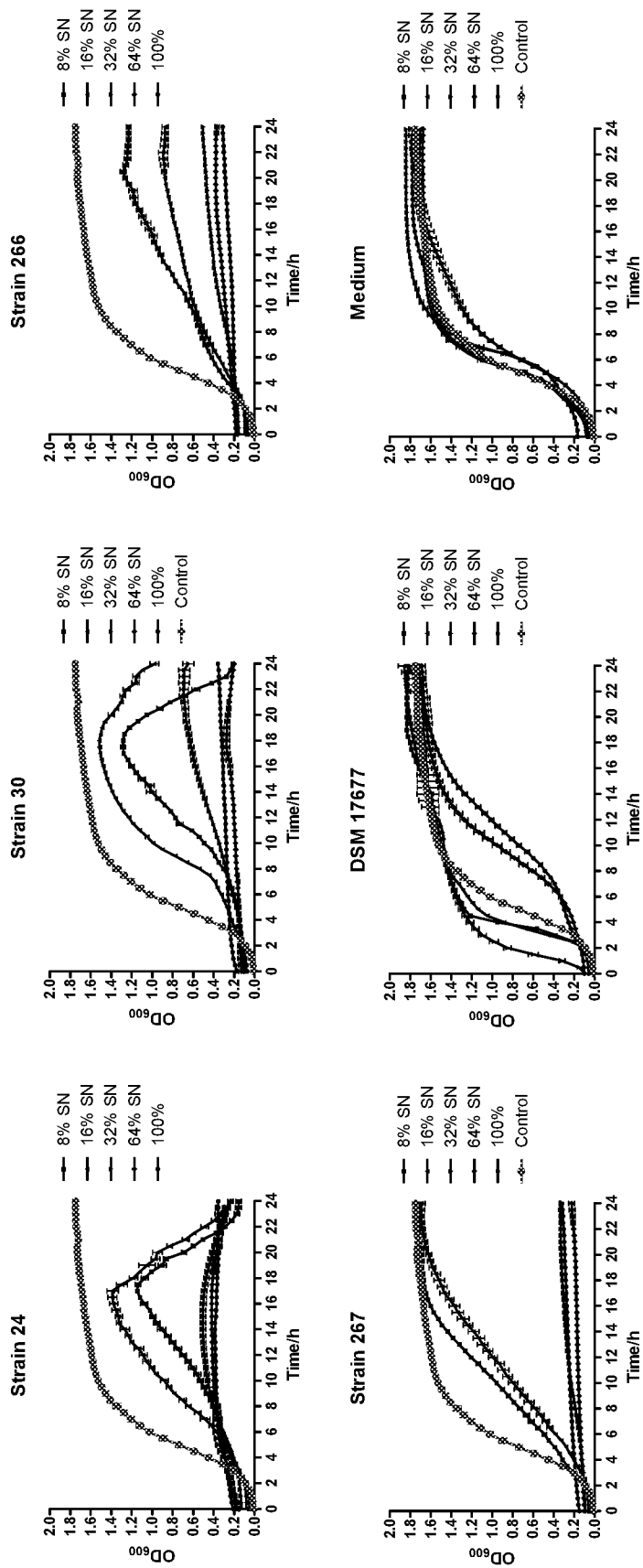


FIG. 5

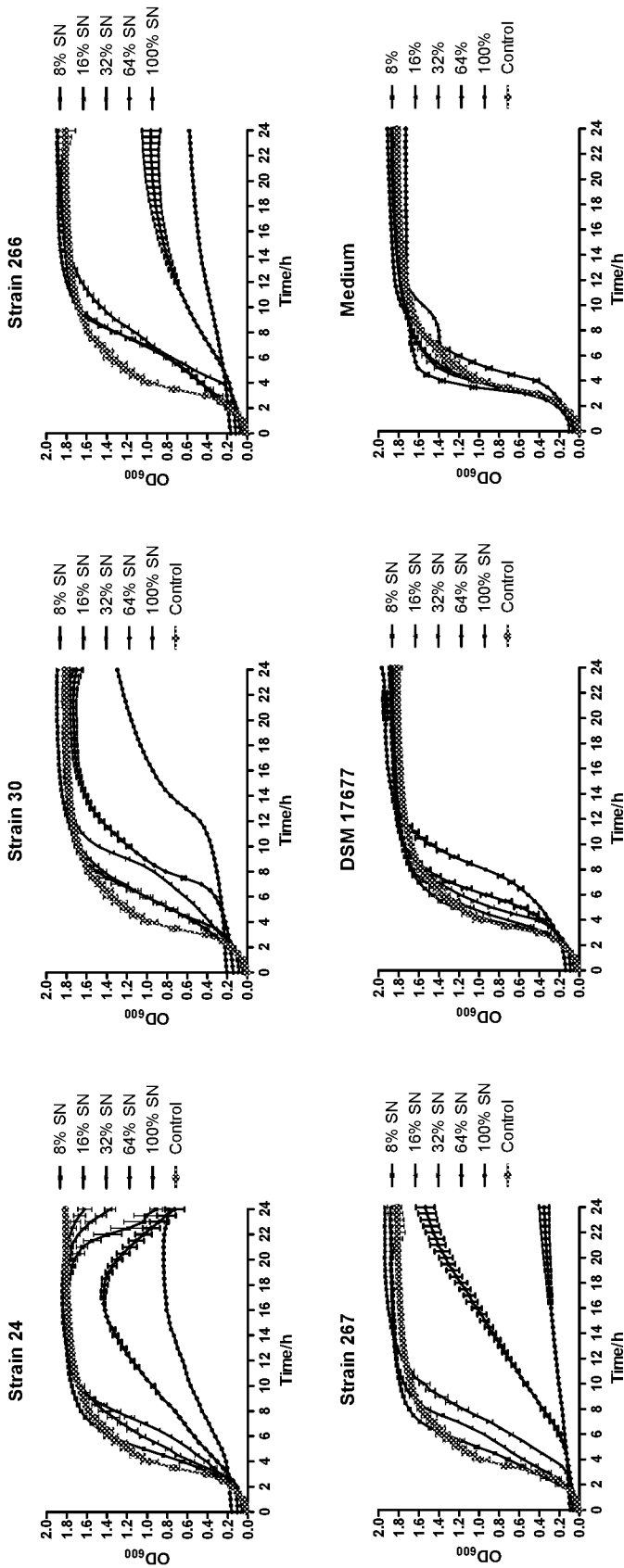


FIG. 6

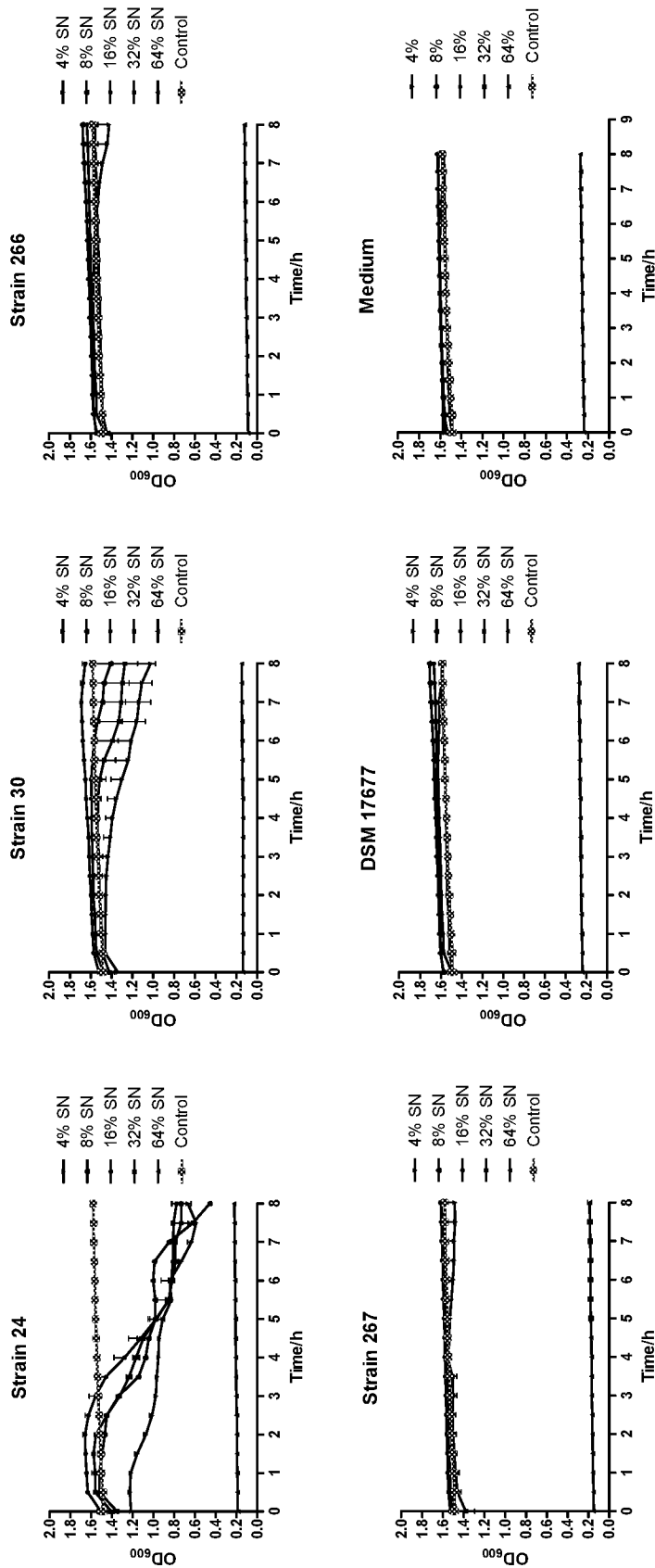
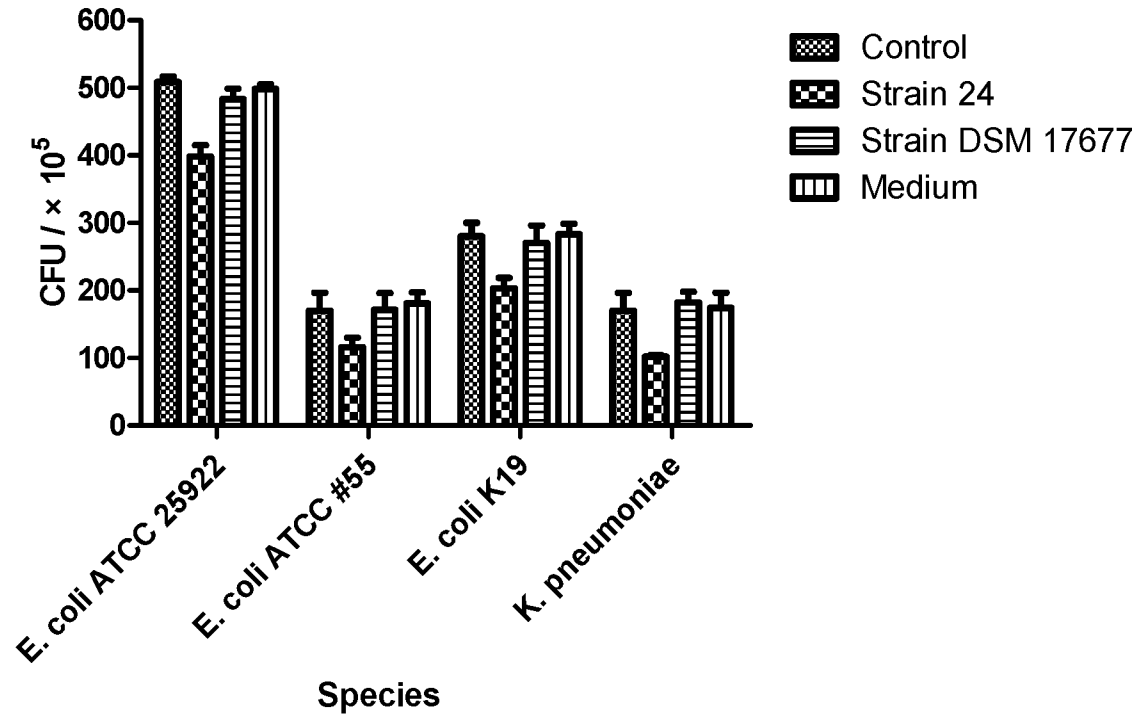


FIG. 7



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/38468

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A23K 20/189, A23K 50/30, A23K 50/75 (2018.01)

CPC - A23K 20/189, A23K 40/30, A23K 50/30

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                               | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | Foditsch et al., "Oral Administration of Faecalibacterium prausnitzii Decreased the Incidence of Severe Diarrhea and Related Mortality Rate and Increased Weight Gain in Preweaned Dairy Heifers", PLoS ONE 10(12): e0145485. doi:10.1371/journal.pone.0145485, 28 December 2015 (28.12.2015); entire document, especially abstract, pg 2 para 1-2, pg 10 para 3 | 1-5, 23-27            |
| X         | US 2016/0243172 A1 (SERES THERAPEUTICS, INC.) 25 August 2016 (25.08.2016); entire document, especially [0008], [0061], [0081]                                                                                                                                                                                                                                    | 1-2, 23-24            |
| A         | Teixeira et al., "Effect of crofelemer extract on severity and consistency of experimentally induced enterotoxigenic Escherichia coli diarrhea in newborn Holstein calves", J Dairy Sci. 2015 Nov;98(11):8035-43. doi: 10.3168/jds.2015-9513. Epub 20 August 2015 (20.08.2015); entire document, especially abstract                                             | 1-5, 23-27            |
| A         | US 2015/0044172 A1 (Cornell University) 12 February 2015 (12.02.2015); entire document                                                                                                                                                                                                                                                                           | 1-5, 23-27            |
| X, P      | US 2017/0348360 A1 (Crestovo Holdings LLC) 07 December 2017 (07.12.2017); entire document                                                                                                                                                                                                                                                                        | 1-5, 23-27            |

☐ 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

"E" earlier application or patent but published on or after the international filing date

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"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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

14 August 2018

Date of mailing of the international search report

18 SEP 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents  
P.O. Box 1450, Alexandria, Virginia 22313-1450

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 18/38468

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 6-22, 28-47  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.