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(71) Applicant: **DEERLAND PROBIOTICS & ENZYMES, INC.** [US/US]; 3800 Cobb International Blvd NW, Kennesaw, GA 30152 (US).

(72) Inventor: **DEATON, John**; 2047 Winsburg Drive, Kennesaw, GA 30144 (US).

(74) Agent: **CARRERA, George, M., Jr.** et al.; AMIN TALATI WASSERMAN LLP, 549 W. Randolph St., Suite 400, Chicago, IL 60661 (US).

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(54) Title: BACILLUS COAGULANS STRAIN, COMPOSITIONS THEREOF, AND METHODS OF USE

(57) Abstract: The invention is directed to new *Bacillus coagulans* strain, which alone or in combination with other *Bacilli* strains can be used as probiotics or together with a prebiotic and a symbiotic. The invention also relates to a composition such as a pharmaceutical composition, dairy product, functional food, nutraceutical, dietary supplement, and product for personal care comprising the new *Bacillus coagulans* strain alone or in combination with other strains, as well as use of the strain for prevention or treatment gastrointestinal infections and diseases, and other uses.

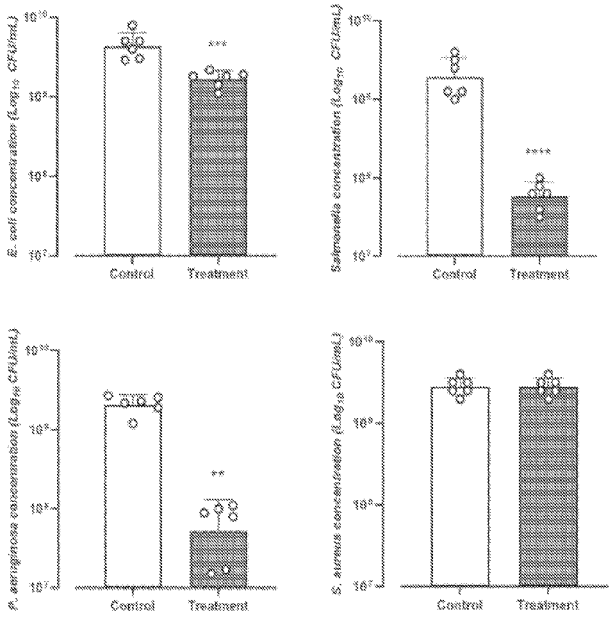

FIG. 4

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BACILLUS COAGULANS STRAIN, COMPOSITIONS THEREOF, AND METHODS OF USE

FIELD OF THE INVENTION

[0001] This invention relates to a new *Bacillus coagulans* strain, which alone or in combination with other *Bacilli* strains can be used as probiotics or together with a prebiotic and a symbiotic. The invention also relates to a composition such as a pharmaceutical composition, dairy product, functional food, nutraceutical, dietary supplement, and product for personal care comprising the new *Bacillus coagulans* strain alone or in combination with other strains, as well as use of the strain for prevention or treatment gastrointestinal infections and diseases, and other uses.

BACKGROUND OF THE INVENTION

[0002] Probiotics are live microorganisms or microbial mixtures administered to improve the patient's microbial balance, particularly the environment of the respiratory and gastrointestinal tract. *Bacillus* strains have been employed for the treatment of respiratory infections, prevention of diarrhoea, as well as, for the treatment of immuno-related diseases (Elshaghabee et al., 2017).

[0003] The normal intestinal flora is dominated by various bacterial species, which produce substances that help control the growth of pathogens. Dysbiosis is a condition that is characterized by a decrease of the certain bacterial species and an increased growth of pathogenic bacteria. Dysbiosis has been associated with the development of periodontal disease, inflammatory bowel disease, and chronic fatigue syndrome. Some studies have suggested patients with dysbiosis may have an increased risk of developing metabolic and cardiac disorders (Chan et al., 2013).

[0004] By administering probiotic *Bacilli*, it is possible to regenerate the intestinal flora of men and women with recurrent episodes of dysbiosis. Dysbiosis is a common gastrointestinal problem. Dysbiosis caused by *Escherichia coli* is also a common problem (Chan et al., 2013).

[0005] The presence of *Bacilli* is important for the maintenance of the intestinal microbial ecosystem. *Bacilli* have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella spp*. and others (Yilmaz et al., 2005). This inhibition could be due to the production of inhibitory compounds such as organic

acids, hydrogen peroxide, bacteriocins or reuterin or to competitive adhesion to the epithelium (Abriouel et al., 2010).

[0006] *Bacilli* have also been examined as a treatment of respiratory tract infections (Marseglia et al., 2007). For example, the installation of *Bacilli*, and stimulation of indigenous organisms has been employed to prevent recurrence of urinary tract infections (Marseglia et al., 2007). The role of *Bacilli* in preventing intestinal infections has also been investigated.

DESCRIPTION OF RELATED ART

[0007] The importance of *Bacilli* as probiotics has been described in the literature.

[0008] Hyronimus et al., 2000 discloses the screening of probiotic activities of a number of *Bacilli* strains by *in vitro* techniques and evaluation of the colonization ability of thirteen selected strains in humans. The strains were examined for resistance to pH 2.5 and 0.3% Oxgall adhesion to Caco-2 cells and antimicrobial activities against enteric pathogenic bacteria (Khochamit et al., 2015). *Bacilli* have been shown to possess the primary requirement of GIT stress tolerance, besides having good adhesion and bio-therapeutic properties (Thakur et al., 2016).

[0009] Pharmaceutical compositions of *Bacilli* known in the art are not sufficiently efficient in recolonizing *in vivo* i.e., mammalian microbial ecosystems and there is, therefore, a need to find *Bacilli* with an inherent ability to recolonize upon administering the *Bacilli* in the form of a pharmaceutical composition, a nutraceutical, a dairy product, a functional food or absorbent product. *Bacilli* isolated from soil, may have the ability to recolonize *in vivo* upon administration because of their inherent ability to survive in the human microbial ecosystem. It is often a cumbersome process to identify *Bacilli* strains with enhanced abilities to colonize upon administration and it is therefore important to select the best test systems to predict their *in vivo* ability to colonize.

[0010] In the literature, there seems to be a large variation in the reported *in vitro* adherence of probiotic strains. This variation indeed reflects biological differences between strains, but certainly also depends on experimental conditions. Moreover, there also seems to be variation with regard to how to measure the adherence. It may be argued that an *in vitro* experiment only serves as a means to estimate the *in vivo* ability to colonize by adherence to epithelial cells.

[0011] Despite being long considered soil microorganisms, *Bacillus spp.* have been used for more than 50 years in the form of fermentation products or spore-based supplements (Cutting et al.,

2011). *Bacilli,* being ubiquitous in nature, consistently enter the gastrointestinal and respiratory tracts of healthy people through food, water, and air (Benno & Mitsuoka, 1986). They have been isolated from the gut and can reach up to 10^7 CFU/g and hence are considered to be one of the dominant components of the normal gut microbiota (Lakshmi et al., 2017). More recently, strains of *Bacillus clausii* have been isolated in order to provide more specific functions and its safety has been evaluated. *Bacillus clausii* has been previously used in diarrhoeal patients (Sudha et al., 2013, Horosheva et al., 2014) and children with recurrent respiratory infections (Marseglia et al., 2007) with no adverse events reported. Though the countries and strains are not specified, *Bacillus clausii* has been commercialized in 55 countries around the world (Nista et al. 2004; Gabrielli et al. 2009). The literature review for *Bacillus clausii* showed no adverse events related to the probiotic and the worldwide presence of bacteria in different countries supplements the narrative of its safety for human consumption.

[0012] *Bacillus coagulans* has a long history of use in a variety of foods. There have been many strains of *Bacillus coagulans* that have been widely consumed around the world for decades (Endres et al., 2009). The presence of this bacterium can be found in foods such as yogurts, milk, sauerkraut, kimchi, and other dairy products, all of which contain levels of *Bacillus coagulans* from 5 x 10^9 CFU/g (Sudha et al., 2016) to 9.38 x 10^{10} (Endres et al., 2011). Several *Bacillus* species have been reported to be common in honeys and include *Bacillus megaterium, Bacillus coagulans, Bacillus subtilis, Bacillus licheniformis, and Bacillus pumilus* (Alippi, 1995; Alippi et al., 2004; Gilliam, 1979; Gilliam & Valentine, 1976; Snowdon & Cliver, 1996). The origin of this bacterial species for use in probiotics stems from India, where a number of manufacturers produce *Bacillus coagulans* as a food ingredient for export and relabeling in Europe and the US (Cutting, 2011). In order to assess the safety of *Bacillus coagulans,* the genome was investigated and it was found that it did not contain any deleterious genes (Salvetti et al., 2016). Due to the nonhazardous nature of *Bacillus coagulans,* this bacterium has been reported safe by the US Food and Drug Administration (FDA) and the European Union Food Safety Authority (EFSA) and is on the Generally Recognized as Safe (GRAS) and Qualified Presumption of Safety (QPS) list (European Food Safety Authority, 2017). Some of the non-pathogenic strains among the 100 known *Bacillus* spp., including *Bacillus coagulans* and *Bacillus subtilis*, were stated as safe for human consumption for all ages (Nithya & Halami, 2012). A literature review for *Bacillus coagulans*

shows no adverse events related to the probiotic which solidifies the findings that is well tolerated and safe. Therefore, *Bacillus coagulans* may be considered a probiotic of safe and consumption providing benefit to the host.

[0013] *Bacillus megaterium* has been found on diverse habitats from soil to seawater, sediment, rice paddies, honey, fish, milk and dried foods (Alfoldi, 1957; Alippi & Reynaldi, 2006; Padgham and Sikora, 2007; Pelletier & Sygusch, 1990; Vary et al., 2007; Von Tersch and Carlton, 1983; Scholle et al., 2003, Kotb, 2014). Further qualitative analysis of microorganisms isolated from honeys revealed that one of the most frequent species of *Bacillus* is *Bacillus megaterium* (Alippi, 1995; Alippi et al., 2004; Snowdon & Cliver, 1996; Tysset, Durand, & Taliergio, 1970). There have been supplemental studies that have isolated *Bacillus megaterium* in fish (Sumathi et al., 2017). Afrilasari et al., 2015 also successfully isolated *Bacillus megaterium* from catfish digestive tract and identified the strain as PTB 1.4. The nonhazardous nature of *Bacillus megaterium* has landed the bacteria on the Qualified Presumption of Safety (QPS) list (European Food Safety Authority, 2017). *Bacillus megaterium* strain ATCC 14581 has been confirmed through genome-analysis to be nearly identical (>99%) to *Bacillus megaterium* MIT411. Health Canada stated the organism is not hazardous to human health or the environment; and exposure to the environment and Canadians is medium. Therefore, it is concluded that *Bacillus megaterium* strain ATCC 14581 is not harmful to human health or to the environment (Health Canada, 2018).

[0014] In summary, *Bacilli* strains with probiotic capabilities should be able to adhere to other suitable cells, such as the cell line Caco-2 cells. Moreover, it is also desirable that the *Bacilli* strains with probiotic capabilities show *in vitro* inhibitory activity against other bacterial species, produce acid after growth in liquid culture and/or produce hydrogen peroxide.

SUMMARY OF THE INVENTION

[0015] It is an object of the present invention to provide strains and compositions as described throughout this application such as pharmaceutical formulations or absorbent products of suitable probiotic *Bacilli* strains with the desired properties. In an embodiment, the present invention concerns the *Bacillus coagulans* strain CGI314 alone or in combination with other strains such as *Bacilli* strains such as *Bacillus megaterium* strain MIT411 (disclosed and claimed in corresponding PCT Application PCT/US2022/xxxxx claiming priority from Irish Patent Application No. 2021/0211, whose contents are incorporated herein in their entirety) and *Bacillus clausii* strain

CSI08 (disclosed and claimed in corresponding PCT Application PCT/US2022/xxxxx claiming priority from Irish Patent Application No. 2021/0209, whose contents are incorporated herein in their entirety). In an embodiment, these strains have similar or essentially the same advantageous properties e.g. the ability to colonize by adherence to mucosal membranes and which are therefore suited for the treatment or prevention of infections or diseases for instance such as the vaginal, urinary-tract, gastrointestinal, naso-sinal, pharyngeal, esophageal, oral, and/or other areas of the body with e.g. mucosal membranes, as well as, treatment or prevention of infections or diseases of the skin and/or other areas of the body having epithelium; immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health amongst others. such as providing antimicrobial activity including lactic acid, benzoic acid, and succinic acid, anti-inflammatory activity, suppression of pro-inflammatory response, activating and/or provoking immune response eg. by stimulating macrophages, providing immunoprotection, aiding in digestion and/or fermentation for instance in the gut, producing branched amino acides, essential amino acids and group B vitamins, maintaining healthy gut and/or skin including lactic acid benzoic acid, and succinic acid in support of skin health, protection of mucosal and other epithelial tissues from toxic agents, decreasing incidence of loose stools, improving the gut-brain axis, and treating and/or preventing dysbiosis and its effects such as periodontal disease, inflammatory bowel disease, chronic fatigue syndrome, metabolic disorders, cardiac disorders, respiratory trat infections, urinary tract infections, GI infections, and diarrhea; and restoring normal and/or healthy flora. In an embodiment, the present invention allows the use of *Bacillus clausii* strain CSI08 and compositions for use in fecal transplants.

[0016] Gastrointestinal diseases include, but are not limited to treating gastrointestinal irregularity in an individual, wherein the individual has at least one 24-hour episode per month of bowel movements measuring 1 or 2 on the Bristol Stool Scale (i.e. treating constipation; or wherein the individual has at least one 24-hour episode per month of bowel movements measuring 6 to 7 on the Bristol Stool Scale (tending towards diarrhoea), wherein the frequency of the individual's 24-hour episodes per month of bowel movements measuring 1 or 2 (or 6 to 7) on the Bristol Stool Scale decreases.

[0017] Also included is a method of restoring gastrointestinal regularity in an individual, wherein the individual has at least one 24-hour episode per month of bowel movements measuring 1 or 2;

or 6 to 7 on the Bristol Stool Scale, wherein the frequency of 24-hour periods of the individual's bowel movements measuring from 3 to 5 on the Bristol Stool Scale increases.

[0018] The invention further includes maintaining healthy gut microflora, with *Bacillus*-containing composition(s). The *Bacillus*-containing composition(s) can be used as probiotic supplementation of the gastrointestinal microflora, and may compete with or otherwise discourage pathogenic bacteria in the gut such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp.

[0019] Another object of the present invention is to provide pharmaceutical formulations with an increased ability to colonize by adherence to the mucosal membrane by employing mucous adhesive excipients.

[0020] It is a further object of the present invention to provide vaginal formulations with an increased ability to suppress the growth of *Candida albicans* and Gram-negative pathogenic bacteria.

[0021] It is yet another objective of the present invention to provide compositions such as dairy products, nutraceutical products and functional foods comprising *Bacillus coagulans* strain CGI314 alone or combination with other *Bacilli* strains such as a *Bacillus megaterium* strain and/or a *Bacillus clausii* strain, having essentially the same properties having the ability to colonize the mucosal membranes and therefore adapted to treatment or prevention of vaginal infections, urinary-tract infections and gastrointestinal diseases. Compositions of the present invention may be administered for 1 dose, 1 day, 1 day to 1 week, 1 day to 1 month, 1 month to 45 days, 45 days to 2 months, 3 months, 6 months, 1 year, or more, including any timeframe identified and/or falling within these ranges.

[0022] The present invention includes methods of treatment and/or preventions and various other methods, which may comprise the steps of providing a *Bacillus coagulans* strain or composition of this invention to a subject (for instance a mammal subject, a human including a human patient, and the like), and administering the strain or composition to the subject. In an embodiment, the present invention is directed to a composition, for instance use of a composition, to treat a disease or infection or other condition. In an embodiment, the present invention is directed to the use of a *Bacillus coagulans* strain or composition thereof, as described throughout this application (including its claims), in the manufacture of a medicament for the treatment of vaginal infections, urinary tract infections, gastrointestinal diseases, improving immune health, protection against

oxidative stress, cleansing and detoxification, metabolic health, cardiovascular health, and/or skin health, and/or other treatments or other reasons for application described throughout this application.

FIGURES

[0023] In the drawings:

[0024] Figure 1 illustrates the phylogenetic tree (16S) of *Bacillus* spp, arranged in clades.

[0025] Figure 2 illustrates the phylogenetic tree (*gyrB*) of *Bacillus* spp., arranged in clades.

[0026] Figure 3 shows antimicrobial activity of *B. coagulans* CGI314 against gut, skin and urinary tract opportunistic pathogens on MRS agar plates with 0.4% TSA agar overlays (solid media). A - *E. coli*; B - *S. enteritidis* and C- *S. aureus*.

[0027] Figure 4 shows *B. coagulans* CGI314 antimicrobial activity in liquid TSB media against gut, skin and urinary tract opportunistic pathogens. Pathogens: *E. coli*, *Salmonella*, *Pseudomonas aeruginosa* and *S. aureus*. (Concentration Log_{10} CFU/ml)

[0028] Figure 5 shows the strongest antimicrobial activity observed with *B. coagulans* CGI314 (Fortispore) against *E. coli* at 24hr. (a) *B. coagulans* MTCC5856 (Lactospore®) (b) *B. coagulans* CG314 (Fortispore) (c) *B. coagulans* 6086 (BC30™) inoculated on MRS agar against *E. coli* 0.4% TSA agar overlay plates.

[0029] Figure 6 shows the strongest antimicrobial activity observed with Fortispore against *S. enteritidis* at 48hr. (a) Lactospore® (b) Fortispore (c) BC30™ inoculated on MRS agar against *S. enteritidis* 0.4% TSA agar overlay plates.

[0030] Figure 7 shows no antimicrobial activity was detected across *Bacillus coagulans* against *P. aeruginosa* (a) *B. coagulans* MTCC5856 (Lactospore®) (b) *B. coagulans* CG314 (c) *B. coagulans* 6086 (BC30™) inoculated on TSA agar against *P. aeruginosa* 0.4% TSA agar overlay plates at 24hr.

[0031] Figure 8 shows limited antimicrobial detected across *B. coagulans* against *S. aureus* using MRS agar at 24 hr. (a) *B. coagulans* inoculated on TSA agar against *S. aureus* 0.4% TSA agar overlay plates. (b) *B. coagulans* inoculated on MRS agar against *S. aureus* 0.4% TSA agar overlay plates.

[0032] Figure 9 shows total antioxidant capacity of *B. coagulans* (Fortispore) and *L. rhamnosus*.

[0033] Figure 10 shows total antioxidant capacity of DE111, *B. coagulans* (Fortispore), *B. clausii* CSI08 (Munispore), *B. megaterium* MIT411 (Renuspore) and *L. rhamnosus*.

[0034] Figure 11 shows adherence of Fortispore to the HT-29 cell line, measured as percentage of adherence bacteria, was negligible.

[0035] Figure 12 shows adherence of Fortispore to the HT-29-MTX cell line, measured as percentage of adherence bacteria, is negligible.

[0036] Figure 13 shows a study on the adherence of *B. coagulans* strains to the HT-29 cell line.

[0037] Figure 14 show a study on the adherence of *B. coagulans* strains to the HT-29-MTX cell line.

[0038] Figure 15 shows the absence of caseolytic activity in *B. coagulans* CGI314 using both streak and overnight TSB broth method at 24h or 48 h.

[0039] Figure 16 shows proteolytic activity of Fortispore towards casein derivatives is lower than positive control Proteinase K using EnzCheck® kit following incubation at 37°C for 24h.

[0040] Figure 17 shows absence of caseolytic activity across (left to right) *B. coagulans CGI314, B. coagulans 6086* (BC30™) and *B. coagulans* MTCC5856 (Lactospore®) using both streak and overnight TSB broth method at 24h or 48h.

[0041] Figure 18 shows Fortispore *B. coagulans* showing low protease activity towards casein.

[0042] Figure 19 shows a quantitative analysis of the caseolytic activity across *B. coagulans* strains determined by EnzCheck® kit following incubation at 37 °C for 24h.

[0043] Figure 20 shows FAA profile of Fortispore UTH fermented milk. Relative concentrations of Methionine, Alanine, Proline, Tryptophan, Lysine, cis-Aconitic acid are shown (white bar (left) represents Control).

[0044] Figure 21 shows FAA profile of Fortispore UTH fermented milk. Relative concentrations of Succinic acid, Lactic acid, Benzoic acid, Isocitric acid are shown (white bar (left) represents Control).

[0045] Figure 22 compares Fortispore with Lactospore and BC30 in the production of Lactic acid, Succinic acid, and Benzoic acid (left to right: Control, BC30, Lactospore, Fortispore).

[0046] Figure 23 compares Fortispore with Lactospore and BC30 in the production of amino acids (left to right: Control, BC30, Lactospore, Fortispore; also, showing relative concentrations of Methionine, Proline, Tryptophan, Lysine).

[0047] Figure 24 shows Fibersol® (F) significantly increased the concentration (CFU/mL) of Fortispore by 1 $\log_{10}$ in minimal media 24 hours post incubation compared to controls.

[0048] Figure 25 shows Fibersol® (F) significantly increases the concentration (CFU/mL) of Fortispore in Minimal media whereas no significance in the growth of BC30 and Lactospore were seen.

[0049] Figure 26 shows that Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of Fortispore in TSB media compared to controls.

[0050] Figure 27 shows Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore, BC30, *E. coli* and *Salmonella enteritidis* in TSB media compared to controls.

[0051] Figure 28 shows Fibersol® (F) did not significantly increase the concentration (CFU/mL) of Fortispore in 50% TSB media compared to controls.

[0052] Figure 29 shows Fibersol® (F) significantly increased the yield (CFU/mL) of DE111 by 1 $\log_{10}$ after 24 hours in 50% TSB media.

[0053] Figure 30 shows that Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of Fortispore compared to controls in BHI media.

[0054] Figure 31 shows that Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore and BC30 compared to controls in BHI media.

[0055] Figure 32 shows that Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of Fortispore compared to controls in 50% BHI media.

[0056] Figure 33 shows that Fibersol® (F) did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore and BC30 compared to controls in 50% BHI media.

DETAILED DESCRIPTION OF THE INVENTION

[0057] Genotypic Identification

[0058] The Applicant collaborated with Cornell University (Ithaca NY, USA) for genomic sequencing and identification.

[0059] WGS DNA Composition

[0060] The whole genome sequence (WGS) was carried out by Cornell University, including assembly and annotation. Bioinformatics analysis was completed at Cornell University and at Deerlands Probiotics and Enzymes (Kennesaw, GA, USA). Identifying gyrB gene poymorphism was carried out the the Applicant.

[0061] The *gyrB* gene encodes DNA gyrase subunit B. DNA gyrase negatively supercoils closed circular double-stranded DNA in an ATP-dependent manner to maintain chromosomes in an underwound state. Gene sequencing analysis used the *gyrB* gene polymorphism, a well-established method for species level discrimination of prokaryotes (Bavykin et al., 2014; Wang et al., 2007). The representative genomes ere reviewed and curated by NCBI, and coordinated with the UniProtein Consortium (NCBI, 2016; UniProt, 2016). R package SequinR coupled with the UniProt Consortium analysis was used to compare whole genome sequences (WGS) and *GyrB* sequence of the presently claimed *Bacillus coagulans* strain CGI314 with other reference strains (Tables 1 to 3 below)

[0062] Genotypic, *gyr*B, & 16S rRNA Identification of *Bacillus coagulans* CGI314

[0063] CGI314 was isolated, and the genome was considered successful.

[0064] The genome size (3.0 Mbp) of CGI314 was shorter than that of previously sequenced *B. coagulans* strain (3.4 Mbp) (Upadrasta et al., 2016). The %GC (47.3%) is consistent with that of a previously sequenced *B. coagulans* strain (46.5%) (Upadrasta et al., 2016).

TABLE 1

Whole genome sequencing metrics of CGI314

Strain	Number of contigs	Total length (nt)	GC (%)	N50 (nt)	Average Coverage (x)
Bacillus coagulans CGI314	210	3,028,595	47.30	29,700	71

TABLE 2

Distance matrix of *gyrB* gene

Bacillus coagulans subsp.	ATCC 7050	BC-30	IS-2	MTCC 5856
CGI314	99.9%	95.2%	95.2%	95.2%

TABLE 3

Whole genome sequence comparison

Bacillus coagulans subsp.	Accession No.	% GC	Sequence Length
CGI314	JABBFU000000000.1	47.30%	3,028,595
ATCC 7050	NZ_CP009709.1	46.90%	3,366,995
BC-30	JPSK01000000	46.39%	3,458,616
IS-2	JZDH01000000	46.41%	3,446,692
MTCC 5856	NZ_CP011939.1	46.23%	3,694,837

[0065] 16S rRNA

[0066] Whole genome sequencing (WGS) and 16S rRNA analysis of CGI314, as compared to the four reference strains, exhibited an average nucleotide identity (ANI) score for 16S rRNA of 99.9%

when compared to the B. coagulans strain ATCC 7050. The genome size (3.0 Mbp) and GC content (47.30%) for CGI314 was comparable to the four reference strains.

[0067] Further Deposits and Accession Numbers

[0068] Genome sequence data of *Bacillus coagulans* strain CGI314 (Fortispore) was deposited into NCBI GenBank database, and the genome sequence was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The genome is publicly available, with GenBank Accession Number JABBFU000000000.1 for the strain, and available for instance at the link: https://www.ncbi.nlm.nih.gov/nuccore/JABBFU000000000.1.

[0069] Genome sequence data of *Bacillus clausii* strain CSI08 (Munispore) was deposited into NCBI GenBank database, and the genome sequence was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The genome is publicly available, with GenBank Accession Number JABBNL000000000.1 for the strain, and available for instance at the link: Alkalihalobacillus clausii strain CSI08, whole genome shotgun sequenci – Nucleotide – NCBI (nih.gov).

[0070] Genome sequence data of *Bacillus megaterium* strain MIT411 (Renuspore) was deposited into NCBI GenBank database, and the genome sequence was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The genome is publicly available, with GenBank Accession Number JABBNK000000000.1 for the strain, and available for instance at the link: Priestia megaterium strain MIT411, whole genome shotgun sequencing pro – Nucleotide – NCBI (nih.gov).

[0071] Phylogenetic Placement, carried out by Deerland Probiotics and Enzymes, Inc.

[0072] Genome-to-genome distance calculation (GGDC), a digital gold standard, is as reliable as DNA-DNA hybridization (DDH) (Auch et al. 2010). GGDC holds more discriminatory power for subspecies delineation and subsequently, was used as a confirmation of multiple alignment and phylogenetic analyses. GGDC yielded calculation-based models that further verified that *Bacillus coagulans* CGI314 is a close relative to ATCC 7050.

[0073] Although the conserved 16S rRNA sequence is a well-established method to compare and study phylogenies in bacteria, the high proportion of sequence similarity between closely related

species limits its usefulness (Wang et al., 2007). High rates of 16S rRNA sequence similarity in closely related bacterial species are due to a slower rate of molecular evolution. Past research (Bavtlin et al., 2004; Wang et al., 2007) supports the validity of using *gyrB* sequences as taxomonic biomarkers due to their rate of base substitutions and significant and reliable correlation with DNA-DNA Hybridization analysis (Dauga et al., 2002; Kasai et al., 1998; Wang et al., 2007). The *gyrB* encodes DNA gyrase B, and type II topoisomerase that plays an important role in DNA replication. Gyrase B subunits are encoded by the *gyrB* gene.

[0074] Phylogenetic analysis using neighbor-joining (NJ) method (Saitou & Nei, 1987) placed *Bacillus coagulans* CGI314 in a clade with *Bacillus coagulans* ATCC 7050 (Figure 2). This confirms all previous genomic identity determinations. *Bacillus coagulans* CGI314 has been placed in the *Bacillus coagulans* group.

DEFINITIONS

[0075] By "excipient" is meant any non-active ingredient that is added to form part of the final formulation.

[0076] By "probiotic" is meant a viable microbial supplement, which has a beneficial influence on the patient through its effects in the intestinal tract, urinary tract or the vaginal tract. The term "probiotic(s)" can refer to live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Foods and food additives containing probiotics may support the restoration of the healthy balance of the gut microflora. Further, probiotic supplementation of the intestinal flora may promote healthy intestinal homeostasis.

[0077] A "prebiotic" is used herein as a substrate, which has a beneficial effect on a probiotic and thus on the individual patient taking the probiotic. Suitable prebiotics may be selected from an inulin, an oligosaccharide, and/or a vitamin.

[0078] A "subject" is used herein includes a person suffering from any clinical condition related to a microbial imbalance as well as a person using bacterial preparations prophylactically. Optionally, the subject is a human.

[0079] By a "symbiotic product" is meant a combination of probiotic and prebiotic, which is synergy, have a beneficial influence on the patient.

[0080] By "hardy growth" is meant that bacteria show excellent growth.

[0081] The abbreviation "CFU" means colony forming units.

[0082] The present invention relating to a probiotic *Bacilli* strain capable of regenerating the *in vivo* flora in subjects will become apparent in the progress of the following detailed description.

[0083] According to a first aspect, the present invention comprises *Bacillus coagulans* CGI314 alone or in combination with other probiotic *Bacilli* strains with essentially the same properties. Such other probiotic *Bacilli* stains may include, but are not limited to a *Bacillus clausii* strain and a *Bacillus megaterium* strain. Such other *Bacilli* strains may further include a *Bacillus clausii* strain and a *Bacillus megaterium* strain each filed today under these respective titles – their contents are incorporated herein in their entirety.

[0084] SEQ ID NO: 1, as recited in the claims attached hereto, comprises gyrB of *Bacillus coagulans* CGI314.

[0085] SEQ ID NO: 2, as recited in the claims attached hereto, comprises 16S rRNA of *Bacillus coagulans* CGI314.

[0086] SEQ ID NO: 3, as recited in the claims attached hereto, comprises the assembled whole genome sequence of *Bacillus coagulans* CGI314.

[0087] The *Bacillus* strain claimed herein, with reference to at least 97% identity to SEQ ID NO: 1 and / or 2; or to at least 97% identity to SEQ ID NO: 3, has the following properties:

[0088] *Bacillus coagulans* CGI314

[0089] The strain shows bile stability.

[0090] The strain shows acid stability.

[0091] The strain show heat tolerance.

[0092] The strain produces a natural antibiotic substance in the form of a bacteriocin.

[0093] In order to determine the genus and species of the strains disclosed herein, the whole genome was sequenced. The amount and composition of the strains were identified and determined.

[0094] The strain was shown to possess little to no antibiotic resistance and no safety concerns.

[0095] The strain was found to show stability toward acid and bile.

[0096] According to a second aspect, the *Bacilli* strain of the present invention is suitable for medical use in preventing or treating vaginal infections, urinary tract infections and gastrointestinal diseases (including gastrointestinal infections), as well as, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health.

[0097] In another preferred embodiment, a pharmaceutical composition is provided comprising *Bacillus coagulans* CGI314 alone or in combination with other probiotic *Bacilli* strains with essentially the same properties, together with a pharmaceutically acceptable carrier and/or diluent. Such other probiotic *Bacilli* stains include, but are not limited to a *Bacillus clausii* strain and a *Bacillus megaterium* strain. The bacterial strains are formulated into pharmaceutical formulations in order to allow the easy administration of the probiotic strains and by means known to the man skilled in the art.

[0098] *Bacillus coagulans* has been proven able to alleviate symptoms of irritable bowel syndrome (Sudha et al., 2018), improve muscle integrity and cytokine response (Gepner et al., 2017; Jager et al., 2018), modulate the gut microbiome and the immune response (Kimmel et al., 2010), reduce function intestinal gas symptoms (Kalman et al., 2009), reduce the instance and duration of diarrhea (Dolin et al., 2009), improve the symptoms of functional abdominal pain and bloating (Hun et al., 2009), protect against acetaminophen induced acute liver injury (Neag et al., 2020), enhance butyrogenesis (Sasaki et al., 2020), reduce severity of bacterial vaginosis (Sudha et al., 2012), and reduce cholesterol (Sudha et al., 2012) all *in vivo*. *Bacillus coagulans* has also shown to induce immune response and anti-inflammatory action (Jensen et al., 2017), improve plant protein digestion (Keller et al., 2017), adhere to Caco-2 cells (Sharma & Kanwar, 2017), improve colonic microenvironment in patients with ulcerative colitis (Sasaki et al., 2020), reduce the adhesion, cytotoxicity and induction of apoptosis caused by S. typhimurium in HT-29 cells (Kawarizadeh et al., 2019), hydrolyze lactose from whey protein (Liu et al., 2019), and enhancing t-cell response (Baron, 2009) all *in vitro*.

[0099] *Bacillus clausii* has been proven efficacious in preventing recurrent respiratory infections (Marseglia et al., 2007), reducing duration and severity of diarrhoea (Sudha et al., 2019) *in vivo*. *Bacillus clausii* has also been proven capable to produce protein hydrolysates with antimicrobial and antioxidant capacity (Rochin-Medina et al., 2017), protect against acetaminophen induced acute liver injury (Neag et al., 2020), inhibit cytotoxic effects induced by *Clostridium difficile* and *Bacillus cereus* toxins (Ripert et al., 2016) *in vitro*.

[0100] *Bacillus megaterium* has been shown to exert protective effects against oxidative stress both *in vitro* and *in vivo* (Mazzoli et al., 2019). *Bacillus megaterium* has also been shown capable

of adapting and surviving in acid stress conditions and chelating heavy metals *in vitro* (Ferreira et al., 2019).

[0101] Preferably, the probiotic bacteria employed in a pharmaceutical in accordance with the present invention are used in bacterial concentration of 10^6-10^{13}. In an embodiment, the probiotic bacteria employed in this invention are used in bacterial concentration of 10^6-10^{13} CFU (colony forming units), for instance as a daily dose, including any amount or range that is included in said range. In an embodiment, the bacteria are employed in an amount of 10^7-10^{12} CFU, or 10^8-10^{11} CFU, or 10^9-10^{10} CFU, or for instance in an amount of about 10^6, about 10^7, about 10^8, about 10^9, about 10^{10}, about 10^{11}, about 10^{12}, and/or about 10^{13} CFU, and any amount or range including or between said amounts. In an embodiment, a composition of this invention comprises, consists essentially of, consists of, and/or is characterized by about 10^6 - about 10^{13} CFU such as about 10^9 *Bacillus coagulans* CGI314. In an embodiment, a composition of this invention comprises *Bacillus coagulans* CGI314 (for instance about 10^9 CFU) in combination with *Bacillus megaterium* MIT411 and/or *Bacillus clausii* CSI08. In an embodiment, a composition of this invention is orally administered in capsule form. In an embodiment, in a composition and/or use or method of this invention, *Bacillus coagulans* CGI314 is in spore form, or is not in spore form.

[0102] In certain embodiments, compositions comprising *Bacillus coagulans* CGI314 can include one or more dry carriers selected from the group consisting of trehalose, maltodextrin, rice flour, microcrystalline cellulose, magnesium stearate, inositol, fructooligosaccharide, galactooligosaccharide, dextrose, dried dairy products, and the like. In certain embodiments, the dry carrier can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight percentage of from about 1% to about 95% by weight of the composition.

[0103] In certain embodiments, the compositions comprising *Bacillus coagulans* CGI314 can include one or more liquid or gel-based carriers, selected from the group consisting of water and physiological salt solutions, urea, alcohols and derivatives thereof (*e.g.*, methanol, ethanol, propanol, butanol), glycols (*e.g.*, ethylene glycol, propylene glycol), and the like; natural or synthetic flavorings and food-quality coloring agents, all compatible with the organism; thickening agents selected from the group consisting of corn starch, guar gum, xanthan gum, and the like; one or more spore germination inhibitors selected from the group consisting of hyper-saline carriers, methylparaben, guargum, polysorbate, preservatives, and the like. In certain embodiments, the

one or more liquid or gel-based carrier(s) can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight/volume percentage of from about 0.6% to about 95% weight/volume of the composition. In certain embodiments, the natural or synthetic flavoring(s) can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight/volume percentage of from about 3.0% to about 10.0% weight/volume of the composition. In certain embodiments, the coloring agent(s) can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight/volume percentage of from about 1.0% to about 10.0% weight/volume of the composition. In certain embodiments, the thickening agent(s) can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight/volume percentage of about 2% weight/volume of the composition. In certain embodiments, the one or more spore germination inhibitors can be added to the compositions comprising *Bacillus coagulans* CGI314 in a weight/volume percentage of about 1% weight/volume of the composition.

[0104] Delivery System

[0105] Suitable dosage forms include tablets, capsules, solutions, suspensions, powders, gums, and confectionaries. Sublingual delivery systems include, but are not limited to, dissolvable tabs under and on the tongue, liquid drops, and beverages. Edible films, hydrophilic polymers, oral dissolvable films, or oral dissolvable strips can be used. Other useful delivery systems comprise oral or nasal sprays or inhalers, and the like. Suitable dosage forms include tablets, capsules, solutions, suspensions, powders, gums, and confectionaries. Sublingual delivery systems include, but are not limited to, dissolvable tabs under and on the tongue, liquid drops, and beverages. Edible films, hydrophilic polymers, oral dissolvable films, or oral dissolvable strips can be used. Other useful delivery systems comprise oral or nasal sprays or inhalers, and the like.

[0106] For oral administration, probiotics may be further combined with one or more solid inactive ingredients for the preparation of tablets, capsules, pills, powders, granules, or other suitable dosage forms. For example, the active agent may be combined with at least one excipient selected from the group consisting of fillers, binders, humectants, distintegrating agents, solution retarders, absorption accelerators, wetting agents, absorbents, and lubricating agents. Other useful excipients include, but are not limited to, magnesium stearate, calcium stearate, mannitol, xylitol, sweeteners, starch, carboxymethylcellulose, microcrystalline cellulose, silica, gelatin, silicon dioxide, and the like

[0107] In certain embodiments, the components of compositions administered according to the methods of the present disclosure, together with one or more conventional adjuvants, carriers, or diluents, may thus be placed into the form of pharmaceutical compositions and unit dosages thereof. Such forms include: solids, and in particular, tablets, filled capsules, powder and pellet forms; liquids, and in particular, aqueous or non-aqueous solutions, suspensions, emulsions, elixirs; and capsules filled with the same; all for oral use, suppositories for rectal administration, and sterile injectable solutions for parenteral use. Such pharmaceutical compositions and unit dosage forms thereof may comprise conventional ingredients in conventional proportions, with or without additional active compounds or principles, and such unit dosage forms may contain any suitable effective amount of the active ingredient commensurate with the intended daily dosage range to be employed.

[0108] The components of the compositions administered according to the methods of the present disclosure can be administered in a wide variety of oral and parenteral dosage forms. It will be obvious to those skilled in the art that the following dosage forms may comprise, in certain embodiments, as the active component, either a chemical compound of the present disclosure or a pharmaceutically acceptable salt of a chemical compound of the present disclosure.

[0109] For preparing pharmaceutical compositions to be administered according to the methods of the present disclosure, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances that may also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or encapsulating materials.

[0110] In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the necessary binding capacity in suitable proportions and compacted in the shape and size desired.

[0111] In certain embodiments, powders and tablets administered according to methods of the present disclosure preferably may contain from five or ten to about seventy percent of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low

melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the active compound with encapsulating material as carrier providing a capsule in which the active component, with or without additional carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid forms suitable for oral administration.

[0112] Liquid preparations include, but are not limited to, solutions, suspensions, and emulsions, for example, water or water-propylene glycol solutions. For example, parenteral injection liquid preparations can be formulated as solutions in aqueous polyethylene glycol solution. In certain embodiments, chemical compounds administered according to methods of the present disclosure may thus be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose for administration in ampoules, pre-filled syringes, small-volume infusion, or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulation agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water, before use.

[0113] Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizing and thickening agents, as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, or other well-known suspending agents.

[0114] Compositions suitable for topical administration in the mouth include, but are not limited to: lozenges comprising the active agent in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base such as gelatin and glycerine or sucrose and acacia; and mouthwashes comprising the active ingredient in suitable liquid carrier.

[0115] Solutions or suspensions are applied directly to the nasal cavity by conventional means, for example, with a dropper, pipette, or spray. The compositions may be provided in single or multi-dose form. In compositions intended for administration to the respiratory tract, including

intranasal compositions, the compound will generally have a small particle size, for example, of the order of 5 microns or less. Such a particle size may be obtained by means known in the art, for example, by micronization.

[0116] The pharmaceutical preparations are preferably in unit dosage forms. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packaged tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself; or it can be the appropriate number of any of these in packaged form.

[0117] Tablets, capsules, and lozenges for oral administration and liquids for oral use are preferred compositions. Solutions or suspensions for application to the nasal cavity or to the respiratory tract are preferred compositions. Transdermal patches for topical administration to the epidermis are preferred.

[0118] Further details on techniques for formulation and administration may be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Publishing Co., Easton, PA).

[0119] In certain embodiments, compositions of the present invention including compositions administered according to the methods of the present disclosure may also include one or more excipients, most preferably one or more nutraceutical or pharmaceutical excipients. Compositions containing one or more excipients and incorporating one or more probiotics can be prepared by procedures known in the art. Optionally, compositions can include one or more adjuvants, excipients, carriers, buffers, diluents, and/or other customary pharmaceutical auxiliaries. For example, probiotics can be formulated into tablets, capsules, powders, suspensions, solutions for oral administration, solutions for parenteral administration including intravenous, intradermal, intramuscular, and subcutaneous administration, and solutions for application onto patches for transdermal application with common and conventional barriers, binders, diluents, and excipients.

[0120] In certain embodiments, nutraceutical compositions including nutraceutical compositions administered according to the methods of the present disclosure may include and may be administered in combination with a pharmaceutically acceptable carrier. In certain embodiments, the active ingredients in such formulations may comprise from about 1% by weight to about 99%

by weight. In other embodiments, the active ingredients in such formulations may comprise from about 0.1% by weight to about 99.9% by weight. "Pharmaceutically acceptable carrier" means any carrier, diluent, or excipient that is compatible with the other ingredients of the formulation and not deleterious to the user. Useful excipients include, but are not limited to, microcrystalline cellulose, magnesium stearate, calcium stearate, any acceptable sugar (*e.g.*, mannitol, xylitol), and the like, and for cosmetic use, a water or an oil base may be used, or mixture thereof including such as an emulsion.

[0121] Routes of Administration

[0122] The strain *Bacillus coagulans* CGI314 or a composition comprising a strain of the present invention may be administered by any route, including, but not limited to, oral, sublingual, buccal, ocular, pulmonary, rectal, vaginal, urethral, ureteral, and parenteral administration, or as an oral or nasal spray (*e.g.*, inhalation of nebulized vapors, droplets, or solid particles). Parenteral administration includes, for example, intravenous, intramuscular, intraarterial, intraperitoneal, intranasal, intravaginal, intravesical (*e.g.*, to the bladder), intradermal, transdermal, topical, or subcutaneous administration. Also contemplated within the scope of the invention is the instillation of a pharmaceutical composition in the body of the patient in a controlled formulation, with systemic or local release of the drug to occur at a later time. For example, the drug may be localized in a depot for controlled release to the circulation, or for release to a local site.

[0123] Pharmaceutical compositions of the invention may be those suitable for, and formulated for, any of the routes identified above, including for instance oral, rectal, bronchial, nasal, pulmonal, topical (including buccal and sub-lingual), transdermal, vaginal, urethral, ureteral, or parenteral (including cutaneous, subcutaneous, intramuscular, intraperitoneal, intravenous, intraarterial, intracerebral, intraocular injection, or influsion) administration, or those in a form suitable for administration by inhalation or insufflation, including powders and liquid aerosol administration, or by sustained release systems. Suitable examples of sustained release systems include semipermeable matrices of solid hydrophobic polymers containing the compound of the invention, which matrices may be in the form of shaped articles, *e.g.*, films or microcapsules.

[0124] The embodiments described above may be further understood in connection with the following Examples. In addition, the following non-limiting examples are provided to illustrate

the invention. However, the person skilled in the art will appreciate that it may be necessary to vary the procedures for any given embodiment of the invention, *e.g.*, vary the order or steps.

EXAMPLE 1

Characterisation of *Bacillus coagulans* CGI314 (referred to as Fortispore hereinafter)

[0125] *B. coagulans* CGI314 (Fortispore) had significant antimicrobial activity against *E. coli, Salmonella* and *S. aureus* in solid MRS agar overlayed with 0.4% TSA agar.

[0126] However, no antimicrobial activity was observed against *P. aeruginosa* in solid media. Fortispore has antimicrobial activity against gut, urinary and skin pathogens in solid environments.

TABLE 4

Summary of *B. coagulans* CGI314 antimicrobial activity against gut, skin and urinary tract opportunistic pathogens (solid media)

Probiotic strain	Zone of inhibition diameter (mm)			
	E. coli	*S. enteritidis*	*S. aureus*	*P. aeruginosa*
Fortispore (CGI314)	28.60 ± 4.10	33.40 ± 4.28	20.67 ± 0.58	-

[0127] Antimicrobial activity is indicated as zone of inhibition (mm) ± standard deviation.

[0128] Figure 3 shows antimicrobial activity of *B. coagulans* CGI314 against gut, skin and urinary tract opportunistic pathogens on MRS agar plates with 0.4% TSA agar overlays (solid media). A - *E. coli*; B - *S. enteritidis* and C- *S. aureus*.

[0129] Fortispore had significant antimicrobial activity against *E. coli, Salmonella* and *P. aeruginosa* in liquid TSB media. However, no antimicrobial activity was observed against *S. aureus* in liquid TSB media.

TABLE 5

Summary of Fortispore antimicrobial activity against gut, skin and urinary tract opportunistic pathogens (liquid media)

Conditions	Inhibition capacity			
	E. coli	*S. enteritidis*	*S. aureus*	*P. aeruginosa*
Solid media	+	+	+	-
Liquid media	+	+	-	+

[0130] Antimicrobial activity detected (+), no antimicrobial activity observed (-).

[0131] Figure 4 shows *B. coagulans* CGI314 antimicrobial activity in liquid TSB media against gut, skin and urinary tract opportunistic pathogens. Pathogens: *E. coli*, *Salmonella*, *Pseudomonas aeruginosa* and *S. aureus*. Control represents growth of pathogen individually; treatment represents growth of pathogen in the presence of *B. coagulans* CGI314. **p<0.01, ***p<0.001, ****p<0.0001.

[0132] Fortispore has potential to control the presence of opportunistic pathogens in the gut and urinary tract where semi-liquid to liquid conditions will be common. Moreover, Fortispore has the potential to prevent the spread of opportunistic pathogens on dryer environments such as the human skin.

[0133] Fortispore has higher antimicrobial activity than the competitor *B. coagulans* probiotics

The antimicrobial activity of *B. coagulans* CGI1314 (Fortispore) was compared with that of *B. coagulans* MTCC5856 (Lactospore®) and *B. coagulans* 6086 (BC30™) using the agar diffusion method against the following opportunistic and zoonotic pathogens of the human skin and the gut:

Escherichia coli 25922,
Salmonella enteritidis 13076,
Staphylococcus aureus RF122, and
Pseudomonas aeruginosa DSM3227.

[0134] *B. coagulans* CGI314 (Fortispore) demonstrated stronger antimicrobial activity against *E. coli* at 24h and *S. enteritidis* at 48h than BC30™ and Lactospore®. Fortispore may have a higher potential to control the presence of opportunistic and zoonotic pathogens that may be present in the gut or on the skin of humans:

High antimicrobial activity of Fortispore against *E. coli.*

High antimicrobial activity of Fortispore against *S. enteritidis* at 48hr.

No Antimicrobial activity detected across *Bacillus coagulans* against *P. aeruginosa.*

Limited antimicrobial activity of *Bacillus coagulans* against *S. aureus* using TSA agar.

[0135] Antimicrobial activity - High antimicrobial activity against *E. coli*:

[0136] *B. coagulans* CGI314 (Fortispore) had the strongest antimicrobial activity against *E. coli* tested using MRS agar. *B. coagulans* MTCC5856 (Lactospore®) and *B. coagulans* 6086 (BC30™) had limited antimicrobial activity.

TABLE 6

Larger zone of inhibition achieved with *B. coagulans* CGI314 (Fortispore) strain against *E. coli*.

	Zone of inhibition diameter (mm) against *E. coli*		
	Rep 1	Rep 2	Rep 3
Fortispore	10 ± 0	9.5 ± 0.7	9 ± 1.4
Lactospore®	2.5 ± 0.7	-	3 ± 0
BC30™	4.5± 0.7	5± 1.4	4.5± 0.7

Values represent average inhibition ± standard deviation (n=2).

[0137] Figure 5 shows the strongest antimicrobial activity observed with *B. coagulans* CGI314 (Fortispore) against *E. coli* at 24hr. (a) *B. coagulans* MTCC5856 (Lactospore®) (b) *B. coagulans* CG314 (Fortispore) (c) *B. coagulans* 6086 (BC30™) inoculated on MRS agar against *E. coli* 0.4% TSA agar overlay plates.

[0138] Fortispore inhibited the growth of the opportunistic intestinal and urinary tract pathogen *E. coli.*

[0139] Antimicrobial activity - High antimicrobial activity of against *S. enteritidis* at 48hr:

[0140] At 24h, *B. coagulans* 6086 (BC30™) had the strongest antimicrobial activity against *S. enteritidis.* However, at 48h, *B. coagulans* CGI314 (Fortispore) had the strongest antimicrobial activity against this pathogen.

TABLE 7

Larger zone of inhibition achieved with *B. coagulans* CGI314 (Fortispore) strain against *S. enteritidis* at 48 hours.

	Zone of inhibition diameter (mm) against *S. enteritidis* at 24 h		
	Rep 1	*Rep 2*	*Rep 3*
Fortispore	6.5 ± 2.1	4.5 ± 0.7	4.5 ± 0.7
Lactospore®	3.5 ± 0.7	-	-
BC30™	10± 0	9± 1.4	6 ± 1.4
	Zone of inhibition diameter (mm) against *S. enteritidis* at 48 h		
Fortispore	11 ± 1.4	13.5 ± 2.12	8 ± 1.4
Lactospore®	4 ± 1.4	-	-
BC30™	10 ± 0	9.5 ± 0.7	7 ± 1.41

Values represent average inhibition ± standard deviation (n=2).

[0141] Figure 6 shows the strongest antimicrobial activity observed with Fortispore against *S. enteritidis* at 48hr. (a) Lactospore® (b) Fortispore (c) BC30™ inoculated on MRS agar against *S. enteritidis* 0.4% TSA agar overlay plates.

[0142] Fortispore inhibited the growth of the zoonotic pathogen *Salmonella* that is known to affect the intestinal tract.

[0143] Antimicrobial activity – no antimicrobial activity detected across *Bacillus coagulans* against *P. aeruginosa*:

[0144] Using the agar diffusion method, no antimicrobial activity was observed across *B. coagulans* against *P. aeruginosa.*

[0145] Figure 7 shows no antimicrobial activity was detected across *Bacillus coagulans* against *P. aeruginosa* (a) *B. coagulans* MTCC5856 (Lactospore®) (b) *B. coagulans* CG314 (c) *B. coagulans* 6086 (BC30™) inoculated on TSA agar against *P. aeruginosa* 0.4% TSA agar overlay plates at 24hr.

[0146] Fortispore does not release antimicrobials against skin pathogen *P. aeruginosa*. However, Fortispore shows potential activity against *P. aeruginosa* in liquid medium (see Table 5 and Figure 7).

[0147] Antimicrobial activity - Limited antimicrobial activity of *Bacillus coagulans* against *S. aureus* using MRS Agar:

[0148] Using TSA agar, there was no evidence of antimicrobial activity by *B. coagulans* strains against the *S. aureus.* However, *B. coagulans* 6086 (BC30™) and *B. coagulans* CGI1314 (Fortispore) did demonstrate slight activity against this pathogen using MRS agar.

TABLE 8

Narrow zones of inhibitions were detected across the three *B. coagulans* strains against *S. aureus* at 24 hours using MRS agar.

	Zone of inhibition diameter (mm) against *S. aureus*		
	Rep 1	*Rep 2*	*Rep 3*
Fortispore	3.5 ± 0.7	2.5 ± 0.7	4.5 ± 0.7
Lactospore®	-	-	-
BC30™	4± 1.4	3± 1.4	5.5± 2.1

Values represent average inhibition ± standard deviation (n=2).

[0149] Figure 8 shows limited antimicrobial detected across *B. coagulans* against *S. aureus* using MRS agar at 24 hr. (a) *B. coagulans* inoculated on TSA agar against *S. aureus* 0.4% TSA agar overlay plates. (Left to right: BC30, CGI314, Lactospore). (b) *B. coagulans* inoculated on MRS agar against *S. aureus* 0.4% TSA agar overlay plates. (Left to right: CGI314, BC30, Lactospore).

[0150] Fortispore can release limited amounts of antimicrobials on dry surfaces such as skin against skin pathogen *S. aureus*.

[0151] Fortispore is a potential antioxidant probiotic:

[0152] Total antioxidant activity of Fortispore *B. coagulans* was compared with *L. rhamnosus.* Fortispore has a higher antioxidant activity than *L. rhamnosus*.

[0153] Figure 9 shows total antioxidant capacity of *B. coagulans* and *L. rhamnosus*. Results show average concentration of Trolox equivalents in nmole/ml (n=3) ± standard error .**p-value =0.0014.

[0154] The increased levels of Trolox equivalent concentration in Fortispore can neutralize and scavenge free radicals and prevent oxidative damage to the cells.

[0155] The Fortispore antioxidant activity is the second highest amount of antioxidant activity amongst all Deerland spores.

[0156] The total antioxidant activity of Fortispore *B. coagulans* was compared with *B. megaterium* MIT411, *B. clausii* CSI08, DE111 *B. subtilis* and *L. rhamnosus*. Fortispore has a high amount of antioxidant activity and is higher than *L. rhamnosus*.

[0157] Figure 10 shows total antioxidant capacity of DE111, *B. coagulans of the present invention, B. clausii* CSI08, *B. megaterium* MIT411and *L. rhamnosus*. Results show average concentration of Trolox equivalents in nmole/ml (n=3) ± standard error.

[0158] The increased levels of Trolox equivalent concentration in Fortispore can neutralize and scavenge free radicals and prevent oxidative damage to the cells.

[0159] Fortispore (vegetative, non-spore form) did not adhere to intestinal epithelial cell line.

[0160] The adhesion ability of *Bacillus coagulans* strain (CGI314) to an *in vitro* model of intestinal epithelium was assessed. Adherence of *B. coagulans* strains CGI314 to HT-29 cell line was negligible at 37°C. Adhesion ability of *B. coagulans* CGI314 to the mucus producing cell line HT-29-MTX was negligible at 37°C.

[0161] Figure 11 shows adherence of Fortispore to the HT-29 cell line was negligible.

[0162] Figure 12 shows adherence of Fortispore to the HT-29-MTX cell line is negligible.

[0163] Fortispore strain does not adhere to intestinal mucus or epithelial cells.

[0164] The adhesion ability of various *Bacillus coagulans* strains (CGI314, BC30™, Lactospore®) to an *in vitro* model of intestinal epithelium was assessed. Adherence of *B. coagulans* strains CGI314, BC30™, Lactospore® to HT-29 cell line is negligible at 37°C. Adhesion ability of *B. coagulans* BC30™ to the mucus producing cell line HT-29-MTX is higher than *B. coagulans* CGI314 and Lactospore® at 37°C.

[0165] Figure 13 shows a study on the adherence of *B. coagulans* strains to the HT-29 cell line.

[0166] Figure 14 show a study on the adherence of *B. coagulans* strains to the HT-29-MTX cell line.

[0167] *B. coagulans* strains (liquid culture, non-spore form) do not adhere to intestinal mucus or epithelial cells.

[0168] Fortispore displays low caseolytic activity

[0169] Fortispore was negative for caseolytic activity on Skim milk agar plates. Quantitative analysis of Fortispore caseolytic activity was evaluated by using a commercial kit employing fluorescently tagged casein derivatives. Fortispore displayed low extracellular protease activity.

[0170] Figure 15 shows a study on the absence of caseolytic activity in *B. coagulans CGI1314* using both streak and overnight TSB broth method at 24h or 48 h.

[0171] Figure 16 shows proteolytic activity of Fortispore towards casein derivatives is lower than positive control Proteinase K using EnzCheck® kit following incubation at 37 °C for 24h. Error bars present standard error (n=3).

[0172] Fortispore displays low protease activity towards Casein.

[0173] Amongst Deerland strains, Fortispore displays low extracellular protease activity towards casein derivatives.

[0174] Figure 17 shows absence of caseolytic activity across *B. coagulans CGI1314, B. coagulans 6086* (BC30™*)* and *B. coagulans* MTCC5856 (Lactospore®) using both streak and overnight TSB broth method at 24h or 48 h.

[0175] Figure 18 shows Fortispore *B. coagulans* showing least protease activity towards casein amongst Deerland strains.

[0176] Figure 19 shows a quantitative analysis of the caseolytic activity across *B. coagulans* strains determined by EnzCheck® kit following incubation at 37 °C for 24h. Significance is indicated by a, b and /or c, multiple letters indicate results are not significantly different from more than one group ($P < 0.05$). Error bars present standard deviations.

[0177] In comparison to the comparators (Lactospore® and BC30™), Fortispore displayed superior protease activity towards casein.

[0178] Fortispore's diverse carbohydrate profile

[0179] Fortispore can metabolize a range of monosaccharides, disaccharides, sugar alcohols, amine sugars and polysaccharides.

[0180] Fortispore was positive for metabolism of 22 carbohydrates out of 49 tested using commercial API 50 CH strip. The majority of these carbohydrates were simple sugars such as D-

Ribose, D-Glucose, D-galactose, D-Fructose, D-Mannose, and Disaccharides such as Trehalose, Maltose and Cellobiose etc. Additionally, there were compounds belonging to glycosylated hydroquinone (arbutin), cyanogenic glycoside (amygladin) and alcoholic β-glucoside (Salicin) that Fortispore can metabolise.

TABLE 9

List of Carbohydrates that are effectively fermented by *B. Coagulans* (Fortispore, BC30™ ,Lactospore®) using API 50 Ch strips

Carbohydrate	*B. coagulans* CGI1314	Carbohydrate	*B. coagulans* CGI1314
L-ARABINOSE	+	AMYGDALIN	+
D-RIBOSE	+	ARBUTIN	+
D-XYLOSE	+	ESCULINFERRIC CITRATE	+
D-GALACTOSE	+	SALICIN	+
D-GLUCOSE	+	D-CELLOBIOSE	+
D-FRUCTOSE	+	D-MALTOSE	+
D-MANNOSE	+	D-MELIBIOSE	-
L-RHAMNOSE	-	D-TREHALOSE	+
D-MANNITOL	+	AMIDON	+
D-SORBITOL	+	GENTIOBIOSE	+
METHYL-AD-GLUCOPYRANOSIDE	+	D-ARABITOL	(+)
N-ACETYLGLUCOSAMINE	+	POTTASIUMGLUCONATE	+

[0181] These data suggest that Fortispore could help digest these compounds in the gut.

[0182] Fortispore was strongly positive for D-sorbitol, and starch Amidon and the other two competitor's strains (BC30™ and Lactospore®) were negative or weakly positive for these.

TABLE 10

List of carbohydrates that are effectively fermented by *B. Coagulans* (Fortispore, BC30™, Lactospore®) using API 50 Ch strips

Carbohydrate	*B. coagulans* 6086 (BC30TM)	*B. coagulans* MTCC5856 (Lactospore®)	*B. coagulans* CGI1314	Carbohydrate	*B. coagulans* 6086 (BC30TM)	*B. coagulans* MTCC5856 (Lactospore®)	*B. coagulans* CGI1314
I-ARABINOSE	+	+	+	AMYGDALIN	+	+	+
D-RIBOSE	+	+	+	ARBUTIN	+	+	+
D-XYLOSE	+	+	+	ESCULIN FERRIC CITRATE	+	+	+
D-GALACTOSE	+	+	+	SALICIN	+	+	+
D-GLUCOSE	+	+	+	D-CELLOBIOSE	+	+	+
D-FRUCTOSE	+	+	+	D-MALTOSE	+	+	+
D-MANNOSE	+	+	+	D-MELIBIOSE	+	+	-
L-RHAMNOSE	+	+	-	D-TREHALOSE	+	+	+
D-MANNITOL	-	+	+	AMIDON	(+)	-	+
D-SORBITOL	(+)	-	+	GENTIOBIOSE	+	+	+
METHYL-AD-GLUCOPYRANOSIDE	+	+	+	D-ARABITOL	-	(+)	(+)
N-ACETYLGLUCOSAMINE	+	+	+	POTTASIUM GLUCONATE	+	+	+

[0183] These data suggest that Fortispore could help digest these compounds in the gut.

[0184] Fortispore has esterase, peptidase, phosphatase and glucosidase activity:

[0185] Fortispore was positive for esterase, peptidase, phosphatase, and galactosidase activity using API ZYM kit which implies:

a high possibility of Fortispore to generate free fatty acids from the action of esterase in the presence of an appropriate lipid source.

Galctosidase adds to the carbohydrate catabolism potential of Fortispore as these are active against various oligosaccharides, lactosylceramides, lactose, and numerous glycoproteins.

Aminopeptidases which catalyze the hydrolysis of leucine, valine and cysteine substrates.

[0186] Indeed, *in silico* analysis have identified genes encoding esterases, peptidases, and galactosidases.

TABLE 11

Enzymatic profile of Fortispore using API ZYM kit

		B. coagulans CGI314 (Fortispore)
Esterase activity	Esterase (C4:0)	+
	Esterase (C8:0)	+
Lipase activity	Lipase (C14:0)	-
Peptidase activity	Leucine arylamidase	+
	Valine arylamidase	+
	Cystine arylamidase	+
Proteinase activity	Trypsin	-
	α-chymotrypsin	-
Phosphatase activity	Acid phosphatase	+
	Alkaline phosphatase	+
	Phosphohydrolyase	+
Glycosidase activity	α-Galactosidase	+
	β-Galactosidase	+
	β-Glucuronidase	-
	α-Glucosidase	+
	β-Glucosidase	+
	β-Glucosaminidase	-
	α-Mannosidase	-
	α-Fucosidase	-

[0187] This study confirms the hydrolytic abilities of Fortispore towards oligosaccharides and indicates the potential to break down fats and peptides to release free amino acids.

[0188] These data suggest that Fortispore could help digest these molecules in the gut.

[0189] *B. coagulans* enzymatic profile indicates peptidase and esterolytic capability and confirms oligosaccharide degradation capability:

[0190] All *B. coagulans* were positive for esterase, peptidase, phosphatase, and galactosidase activity using API ZYM kit which implies:

a high possibility of *B. coagulans* to generate free fatty acids from the action of esterase in the presence of an appropriate lipid source.

Galactosidase adds to the carbohydrate catabolism potential of *B. coagulans* as these are active against various oligosaccharides, lactosylceramides, lactose, and numerous glycoproteins.

Aminopeptidases which catalyze the hydrolysis of leucine, valine and cysteine substrates.

TABLE 12

Enzymatic profile of Fortispore using API ZYM kit

		B. coagulans CGI1314 (Fortispore)	*B. coagulans* MTCC5856 (Lactospore®)	*B. coagulans* 6086 (BC30™)
Esterase activity	Esterase (C4:0)	+	+	+
	Esterase (C8:0)	+	+	+
Lipase activity	Lipase (C14:0)	-	-	-
Peptidase activity	Leucine arylamidase	+	+	+
	Valine arylamidase	+	+	+
	Cystine arylamidase	+	+	+
Proteinase activity	Trypsin	-	-	-
	α-chymotrypsin	-	-	-
Phosphatase activity	Acid phosphatase	+	+	+
	Alkaline phosphatase	+	+	+
	Phosphohydrolyase	+	+	+
Glycosidase activity	α-Galactosidase	+	+	+
	β-Galactosidase	+	+	+
	β-Glucuronidase	-	-	-
	α-Glucosidase	+	+	+
	β-Glucosidase	+	+	+
	β-Glucosaminidase	-	-	-
	α-Mannosidase	-	-	-
	α-Fucosidase	-	-	-

[0191] This study demonstrates no differences in hydrolytic abilities of *B. coagulans* strains towards several enzymes using a commercial API ZYM Kits.

[0192] Fortispore releases free amino acids from glycolysis and protease metabolism

[0193] Metabolomic analysis reveals Fortispore fermentation and proteolytic capability towards milk carbohydrates and proteins generating a range of amino acids.

[0194] UHT Milk model was used to analyse the carbohydrate fermentation and proteolytic abilities of Fortispore. GC-MS analysis identified a total of 38 free amino acids (FAA) compounds,

of which 10 were found to be statistically significant in Fortispore. A few of the carboxylic acids identified are associated with carbohydrate metabolism pathway, which confirms the presence of a Lactose/ galactose/ glucose uptake and enzymatic metabolism system to be active in Fortispore. Additionally, there is also evidence of an active proteolytic system in Fortispore as there is a release of a few amino acid linked with peptidase activity and benzoic acid which is linked with further catabolism of phenylalanine.

TABLE 13

Statistically significant compounds analysed with GC-MS using the FAA method are listed along with potential precursors

Compound	Pottential peptidases/ precursors
Alanine	Aminopeptidases PepN
Proline	Pep P/ PepX
Methionine	MAP/ PepM
Lysine	Aminopeptidases PepN
Tryptophan	Aminopeptidases
Succinic acid	Lactose/ glucose
Lactic acid	Lactose/ glucose
cis-Aconitic acid	Lactose/ glucose
Isocitric acid	Lactose/ glucose
Benzoic acid	Phenylalanine

[0195] Overall, the enzymatic and metabolomic data suggest that Fortispore has active carbohydrate-metabolizing enzymes, as most of the compounds generated are those from the glycolysis pathway (conversion of carbohydrates in milk). Additionally, there is evidence of potentially active aminopeptidases, lyases and decarboxylases generating carboxylic acids and releasing amino acids from Fortispore fermented UHT milk.

[0196] Fortispore releases free amino acids from glycolysis and protease metabolism

[0197] Free Amino Acid analysis presented in bar graphs (Mean +SEM)

[0198] Figure 20 shows FAA profile of Fortispore UTH fermented milk. P-value ≤ 0.005 = ** and P-value ≤ 0.01 = *. The white bar represents the control.

[0199] Figure 21 shows FAA profile of Fortispore UTH fermented milk. P-value ≤0.005 = ** and P-value ≤0.01 = *. The white bar represents the control.

[0200] Fortispore is slightly more efficient in producing compounds beneficial in skin health in comparison to competitor *B. coagulans* strains

[0201] Ganeden Biotech markets *B. coagulans* 6086 (BC30™) as an anti-aging probiotic ,as it produces maximum amounts of naturally derived L+ lactic acid, bacteriocins, hydrogen peroxide, enzymes and other metabolites. Lactic acid is associated with anti-aging, fighting acne due to its antimicrobial activity and hydration. Succinic acid is also associated with anti-inflammatory, antimicrobial and hydration. Benzoic acid is associated with anti-microbial activity.

[0202] Figure 22 compares Fortispore with Lactospore and BC30 in the production of lactic acid, succinic acid and Benzoic acid. Significance is indicated by a, b and /or c, multiple letters indicate results are not significantly different from more than one group ($P < 0.05$). Error bars present standard deviations.

[0203] Our results show Fortispore to be slightly more efficient than BC30™ in terms of lactic acid production and superior in succinic acid and benzoic acid production.

[0204] Fortispore can produce amino acids beneficial in skin health in comparison to competitor *B. coagulans* strains

[0205] Fortispore is compared with Lactospore® and BC30. Our results show Fortispore to be superior to BC30™ and Lactospore® in producing methionine, proline, lysine and tryptophan.

[0206] Methionine is a sulfur-containing amino acid that improves the tone and elasticity of the skin, promotes healthy hair and strengthens the nails. Proline helps lower inflammation, which promotes a healthy immune system. It also helps trigger a cascade of anti-inflammatory compounds and genes that help with recovery. Lysine is an essential amino acid with many benefits that range from preventing cold sores to reducing anxiety and promoting wound healing. Tryptophan is associated with relief from depression and anxiety.

[0207] Figure 23 compares Fortispore with Lactospore and BC30 in the production of amino acids. Significance is indicated by a, b and /or c, multiple letters indicate results are not significantly different from more than one group ($P < 0.05$). Error bars present standard deviations.

[0208] Fortispore proteomics analysis identifies proteins with potential probiotic benefits

[0209] Extracellular secretions of Fortispore were sent to mass spectrometry to identify proteins released by the probiotic strain. A total of 28 proteins were detected of which 6 had potential probiotic benefits.

TABLE 14

Fortispore proteomics identifies proteins with potential probiotic benefits

Protein of interest	Potential role
Hydrolase Nlp/P60	Involved in degradation of proteins or fats
Serine protease	Involved in digestion of proteins - cleaves peptide bonds
Peptidase M23	Involved in digestion of proteins Antimicrobial properties - lysis of bacterial cell wall
Chaperonin GroEL	Involved in protein folding
Peptidase	Involved in digestion of proteins
Dextransucrase	Involved in breakdown of starch and sucrose

[0210] These data confirm previous *in vitro* results showing how Fortispore can help in digestion of proteins and carbohydrates and has antimicrobial properties against pathogens.

[0211] Fortispore growth increased in the presence of Fibersol® in minimal media.

[0212] Figure 24 shows Fibersol® significantly increased the concentration (CFU/mL) of Fortispore by 1 $\log_{10}$ in minimal media 24 hours post incubation compared to controls. *$p < 0.05$

[0213] There is a significant increase in the growth of Fortispore in the presence of Fibersol® in minimal media in comparison to competitor *B. coagulan* strains.

[0214] Figure 25 shows Fibersol® significantly increases the concentration (CFU/mL) of Fortispore in Minimal media whereas no significance in the growth of BC30 and Lactospore were

seen. Statistical analysis conducted using one–way ANOVA using Tukey method. **p-value =< 0.01.

[0215] There was no increase in the growth of Fortispore in the presence of Fibersol® in TSB media.

[0216] Figure 26 shows that Fibersol® did not show significant increase in the concentration (CFU/mL) of Fortispore in TSB media compared to controls. Unlike minimal media, Fibersol® didn't increase the growth of Fortispore in rich media, likely because they reach maximum growth with the nutrients present in TSB media.

[0217] The growth of Fortispore, BC 30, DE111 and pathogens were compared in the presence of Fibersol® in TSB media.

[0218] Figure 27 shows Fibersol® did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore, BC30, *E. coli* and *Salmonella enteritidis* in TSB media compared to controls. Unlike minimal media, Fibersol® didn't increase the growth of DE111, Fortispore, BC30, *E.coli* and *S. enteritidis* in rich media, most likely because they reach maximum growth with the nutrients present in TSB media.

[0219] There was no increase in the growth of Fortispore in the presence of Fibersol® in 50% TSB media.

[0220] Figure 28 shows Fibersol® did not significantly increase the concentration (CFU/mL) of Fortispore in 50% TSB media compared to controls.

[0221] The growth of Fortispore, BC 30 and DE111 was compared in the presence of Fibersol® in 50% TSB media.

[0222] Figure 29 shows Fibersol® significantly increased the yield (CFU/mL) of DE111 by 1 $\log_{10}$ after 24 hours in 50% TSB media. ****p<0.0001. Fibersol® did not show significant increase in the concentration (CFU/mL) of Fortispore and BC30 in 50% TSB media compared to controls.

[0223] No significant growth of Fortispore was observed in the presence of Fibersol® supplemented BHI media.

[0224] Figure 30 shows that Fibersol® did not show significant increase in the concentration (CFU/mL) of Fortispore compared to controls in BHI media. Unlike minimal media, Fibersol® didn't increase the growth of Fortispore in rich media, most likely because they reach maximum growth with the nutrients present in BHI media.

[0225] The growth of Fortispore, BC 30 and DE111 was compared in the presence of Fibersol® in BHI media.

[0226] Figure 31 shows that Fibersol® did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore and BC30 compared to controls in BHI media. Unlike minimal media, Fibersol® didn't increase the growth of DE111, Fortispore and BC30 in rich media, most likely because they reach maximum growth with the nutrients present in BHI media.

[0227] No increase in the growth of Fortispore was observed in the presence of Fibersol® in 50% BHI media.

[0228] Figure 32 shows that Fibersol® did not show significant increase in the concentration (CFU/mL) of Fortispore compared to controls in 50% BHI media. Unlike minimal media, Fibersol® didn't increase the growth of Fortispore in rich media, most likely because they reach maximum growth with the nutrients present in BHI media.

[0229] No increase in the growth of Fortispore, BC 30 and DE111 was observed in the presence of Fibersol® in 50% BHI media.

[0230] Figure 33 shows that Fibersol® did not show significant increase in the concentration (CFU/mL) of DE111, Fortispore and BC30 compared to controls in 50% BHI media. Unlike minimal media, Fibersol® didn't increase the growth of DE111, Fortispore and BC30 in rich media, most likely because they reach maximum growth with the nutrients present in BHI media.

EXAMPLE 2

Assess adhesion ability to an *in vitro* model of intestinal epithelium

[0231] Cell lines: Human Colorectal Adenocarcinoma Cell Line HT-29 and mucous-secreting cell line HT-29-MTX were propagated using low glucose DMEM medium supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/l streptomycin, and 2 µg/ml amphotericin B in a 5% CO_2 atmosphere at 37°C.

[0232] Cells were seeded onto 24-well plates at a density $5x10^5$ cell/well and cultured for 21-28 days to complete maturation. Media was replaced every 2-3 days.

[0233] Prior to experiments cells were washed twice with 0.5 ml DPBS. DPBS was completely aspirated from the wells after the second round of washing.

[0234] Preparation of spores: Ten milligrams of *B. clausii* CSI08, *B. megaterium* MIT411 and *B. coagulans* CGI314 spores powders were weighted in 15 ml falcon tubes and resuspended in 10 ml of full culture medium without antibiotics. Suspensions were aliquoted and stored at -20°C until use. Suspensions were used within 2 weeks upon preparation.

[0235] Adhesion assay: 500 µl of spores suspensions ($1.3x10^7$ - $9.2x10^7$ CFU/ml) were added to HT-29 and HT-29-MTX cells, mixed by a gentle swirl, and incubated for 2.5 h at 37°C in the CO_2 incubator. Control wells not containing mammalian cells were prepared and incubated in parallel in the same way (0.5 ml of spores' suspensions).

[0236] Upon incubation HT-29 and HT-29-MTX cells were washed 4 times with 0.5 ml PBS. After that 50 µl of Trypsin/EDTA solution and 50 µl of PBS were added to the wells and incubated for 10 min with gentle shaking (~100 rpm) at 37°C. Fifty microliters of Trypsin/EDTA solution were added to control wells.

[0237] Consequently, 450 µl of PBS were added to the wells with spores, contents of the wells were transferred into Eppendorf tubes with scrapping and subjected to three rounds of vigorous shaking 30 sec each. Contents of control wells were transferred into Eppendorf tubes and subjected to one round of shaking.

[0238] Serial dilutions (plus dilutions of control wells) were prepared in PBS and plated onto BC agar (*B. coagulans* CGI314) or PetriFilm™ (*B. clausii* CSI08, *B. megaterium* MIT411). Plates were incubated at 37°C for 48 h prior to counting, PetriFilm were incubated at 37°C for 24 h prior to counting.

[0239] Experiments were performed two or three times with three technical replicates per experiment. The results are expressed as means ± SEM.

TABLE 15

Adherence of *B. clausii* CSI08, *B. megaterium* MIT411 *and B. coagulans* CGI314 spores to the HT-29-MTX cell line

Percentage of adherence to HT-29-MTX cell line	*B. clausii* CSI08	*B. megaterium* MIT411	*B. coagulans* CGI314
Mean	12.10	20.68	19.68
Standard error of the mean	0.7710	3.005	2.197

TABLE 16

Adherence of *B. clausii* CSI08, *B. megaterium* MIT411 *and B. coagulans* CGI314 spores to the HT-29 cell line

Percentage of adherence to HT-29 cell line	*B. clausii* CSI08	*B. megaterium* MIT411	*B. coagulans* CGI314
Mean	0.2578	1.499	0.8033
Standard error of the mean	0.02035	0.2983	0.1781

Conclusion:

1. Results set out above demonstrate higher ability of spores to adhere to the mucous-secreting cell line HT-29-MTX compared to non-mucus secreting cells, possibly due to spores' physical properties.
2. *B. megaterium* MIT411 and *B. coagulans* CGI314 spores have higher (but overall low) ability to adhere to non-mucus producing cell line HT-29 compared to *B. clausii* CSI08 spores.

EXAMPLE 3

Evaluation of *Bacillus coagulans* CGI314 on safety, tolerance and gastrointestinal health: a randomised, double-blind, placebo-controlled trial in healthy adults:

[0240] STUDY INTERVENTIONS

Probiotic 1 containing 1 x 10^9 CFU of *Bacillus coagulans* CGI314 and *Bacillus subtilis* DE111® administered daily,

Probiotic combination containing about (and greater than) 0.5 x 10^9 CFU of *Bacillus Subtilis* DE111®, about 0.5 x 10^9 CFU of *Bacillus megaterium* MIT411, about (and less than) 0.5 x 10^9 CFU *Bacillus coagulans* CGI314, about 0.5 x 10^9 CFU *Bacillus clausii* CSI08; 2x10^9 *Bacillus* spores total administered daily

Placebo : rice maltodextrin administered daily

[0241] INDICATION STUDIED

Gastrointestinal homeostasis and immune system.

[0242] STUDY DESCRIPTION

Healthy adults aged 18-65 years were recruited and screened for participation in the study. Eligible participants were randomized 1:1:1: to either one of the two experimental groups or control group and underwent 45 days of treatment and 2 weeks of follow-up to evaluate safety and efficacy of new probiotic strains in healthy adults.

[0243] For the purpose of this investigational products were packed in individual capsules, that corresponded to the daily dose. Each capsule contained 300 mg of ingredients:

Probiotic formula 1: *Bacillus coagulans* CGI314 and *Bacillus subtilis* DE111® (1 x 10^9 CFU total), ~4.17 mg; Low Moisture Rice Maltodextrin, 292.23mg; Medium Chain Triglycerides, 3.6 mg.

Probiotic cocktail: 2x10^9 CFU *Bacillus* spores, including *Bacillus subtilis* DE111® (about (and greater than) 0.5 x 10^9 CFU), 2.1 mg; *Bacillus megaterium* MIT411 (about 0.5 x 10^9 CFU), 2.1 mg; *Bacillus clausii* CSI08 (about 0.5 x 10^9 CFU), 2.1 mg; *Bacillus coagulans* CGI314 (about (and less than) 0.5 x 10^9 CFU), 2.1 mg; Low Moisture Rice Maltodextrin, 288 mg; Medium Chain Triglycerides, 3.6 mg.

Placebo consisted of all the same ingredients as investigational product only without probiotic organisms. Placebo was in individual capsules, that corresponded to the daily dose. Each capsule contained 300 mg of ingredients: Placebo: Low Moisture Rice Maltodextrin, 296.4 mg; Medium Chain Triglycerides, 3.6 mg.

[0244] Table 17 summarizes the change in scores of the Gut-brain axis questionnaire from baseline to the end of the treatment period, for the 4 probiotic groups and the placebo group. Mean changes with 95 % confidence interval are shown. Results of the ANOVA omnibus test (p*-value) and one-sample T test (p-value) are also presented. Test of normality for the change in scores of the Gut-brain axis show that the data do not follow normal distribution, which could affect the results with borderline significance (p-values between 0.05 and 0.10). This affects two items: Loss of energy and Changes in appetite. An alternative nonparametric Kruskal Wallis test was applied to these items; p-values of 0.111 (Loss of energy) and 0.123 (Changes in appetite) were observed.

[0245] In general, mean values of the score changes of the Gut-brain axis questionnaire are negative for all the symptoms tested, meaning that the symptoms were less intense (participants were less bothered by these symptoms) at the end of the treatment period. One-sample T-test results show that in one third of tests (of 70 performed) a statistically significant change Gut-brain axis questionnaire score was observed. However, this can be observed for all treatment groups including the placebo group. Consequently, the results of the ANOVA test show, that no significant differences in Gut-brain axis score change among the treatment groups were detected, however a borderline significance for the items Loss of energy and Changes in appetite was observed. The participants in the *Bacillus megaterium* group experienced the largest change for these two items.

TABLE 17

Change in scores of the Gut-brain axis questionnaire items from baseline to the end of the treatment period: Pairwise comparison of probiotic groups with placebo (post hoc ANOVA: Dunnet t-test)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)
Sadnesschange		

mean difference vs placebo (95% CI)	-0.42 (-1.00; 0.17)	-0.44 (-1.02; 0.14)
p-value vs placebo	0.236	0.192
Irritationchange		
Mean difference vs placebo (95% CI)	-0.33 (-0.94; 0.27)	-0.35 (-0.95; 0.25)
p-value vs placebo	0.453	0.405
Loss of energychange		
Mean difference vs placebo (95% CI)	0.08 (-0.57; 0.74)	-0.02 (-0.67; 0.63)
p-value vs placebo	0.993	1.000
Changes in apetitechange[a]		
Mean difference vs placebo (95% CI)	-0.33 (-1.01; 0.34)	-0.04 (-0.54; 0.46)
p-value vs placebo	0.390	0.999
Hard to breathe/chokingchange		
Mean difference vs placebo (95% CI)	-0.21 (-0.61; 0.19)	-0.16 (-0.56; 0.24)
p-value vs placebo	0.505	0.709
Heart pounding/racingchange[a]		
Mean difference vs placebo (95% CI)	-0.33 (-0.92; 0.25)	0.08 (-0.35; 0.51)
p-value vs placebo	0.176	0.968
Sleeping problemschange		
Mean difference vs placebo (95% CI)	-0.04 (-0.66; 0.57)	-0.15 (-0.76; 0.46)
p-value vs placebo	0.999	0.926
Concentration problemschange		
Mean difference vs placebo (95% CI)	0.13 (-0.42; 0.67)	-0.02 (-0.56; 0.51)
p-value vs placebo	0.944	1.000
Nervousness/stresschange		
Mean difference vs placebo (95% CI)	0.00 (-0.71; 0.71)	-0.14 (-0.85; 0.56)
p-value vs placebo	1.000	0.963
Angriness/tensionchange		
Mean difference vs placebo (95% CI)	-0.04 (-0.63; 0.54)	-0.35 (-0.93; 0.23)
p-value vs placebo	0.999	0.372
Headachechange		

Mean difference vs placebo (95% CI)	-0.17 (-0.76; 0.42)	-0.03 (-0.61; 0.55)
p-value vs placebo	0.892	1.000
Muscle aches/painchange		
Mean difference vs placebo (95% CI)	0.33 (-0.17; 0.84)	0.13 (-0.37; 0.63)
p-value vs placebo	0.293	0.912
Stiffnesschange		
Mean difference vs placebo (95% CI)	0.08 (-0.33; 0.50)	0.17 (-0.24; 0.58)
p-value vs placebo	0.965	0.692
Dizzinesschange		
Mean difference vs placebo (95% CI)	-0.13 (-0.49; 0.24)	0.00 (-0.36; 0.36)
p-value vs placebo	0.807	1.000

N* = number of participants included in the ITT population, *p-value for Dunnet T-test (pairwise comparison)

[0246] Mean differences with 95% confidence intervals and p-values of pairwise comparison between individual probiotic group and placebo group are shown in Table 17. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in Gut-brain axis score change from baseline to the end of treatment period.

[0247] Mean differences with 95% confidence intervals and p-values of pairwise comparison between individual probiotic group and placebo group are shown in Table 18. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in the sum of Gut- brain axis items scores at baseline or at the end of treatment period.

TABLE 18

The sum of scores of the Gut-brain axis questionnaire items at baseline and at the end of the treatment period: Pairwise comparison of probiotic groups with placebo (post hoc ANOVA: Dunnet t-test)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)
Sum of *Gut brain axis items* scores at baseline		
Mean difference vs placebo (95% CI)	0.43 (-3.86; 4.71)	-0.02 (-4.30; 4.27)
p-value vs placebo	0.997	1.000
Sum of *Gut-brain axis items* scores at the end of the treatment period		
Mean difference vs placebo (95% CI)	-1.00 (-4.62; 2.62)	-1.34 (-4.92; 2.24)
p-value vs placebo	0.899	0.762

N* = number of participants included in the ITT population, *p-value for Dunnet T-test (pairwise comparison)

[0248] Mean differences with 95% confidence intervals and p-values of pairwise comparison between individual probiotic group and placebo group are shown in Table 19. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in HDL, LDL, TC or TG change from baseline to the end of treatment period.

TABLE 19

Cholesterol and triglyceride change from baseline to the end of the treatment period in ITT population: Pairwise comparison of probiotic groups with placebo (post hoc ANOVA: Dunnet t-test)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)
HDL_{change} [mmol/L]		
mean difference vs placebo (95% CI)	0.00 (-0.12; 0.11)	0.00 (-0.12; 0.11)
p-value vs placebo	1.000	1.000
LDL_{change} [mmol/L]		
mean difference vs placebo (95% CI)	-0.05 (-0.34; 0.23)	-0.08 (-0.37; 0.21)

p-value vs placebo	0.972	0.888
TCchange [mmol/L]		
mean difference vs placebo (95% CI)	0.01 (-0.41; 0.43)	-0.01 (-0.43; 0.41)
p-value vs placebo	1.000	1.000
TGchange [mmol/L]		
mean difference vs placebo (95% CI)	-0.17 (-0.62; 0.28)	-0.03 (-0.48; 0.42)
p-value vs placebo	0.762	0.999

N* = number of participants included in ITT population

[0249] Mean differences with 95% confidence intervals and p-values of pairwise comparison between individual probiotic group and placebo group are shown in Table 20. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in HDL, LDL, TC or TG relative change from baseline to the end of treatment period.

TABLE 20

Cholesterol and triglyceride relative change from baseline to the end of the treatment period in ITT population: Pairwise comparison of probiotic groups with placebo (post hoc ANOVA: Dunnet t-test)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)
HDL relative change [%]		
mean difference vs placebo (95% CI)	-0.56 (-7.87; 6.76)	-0.58 (-7.89; 6.74)
p-value vs placebo	0.999	0.999
LDLrelative change [%]		
mean difference vs placebo (95% CI)	-2.45 (-12.19; 7.30)	-4.37 (-14.12; 8.62)
p-value vs placebo	0.926	0.632
TCrelative change [%]		
mean difference vs placebo (95% CI)	0.87 (-6.37; 8.11)	-0.40 (-7.64; 6.84)
p-value vs placebo	0.995	1.000
TGrelative change [%]		

mean difference vs placebo (95% CI)	-5.70 (-35.29; 23.89)	6.40 (-23.18; 35.99)
p-value vs placebo	0.970	0.955

N* = number of participants included in ITT population, *p-value of Dunnet T-test (pairwise comparison)

[0250] Table 21 represents proportion of people who had reported at least one day with clinically relevant infection in Participant diary 1. In Table 22 descriptive statistics of number of days with clinically relevant infection and results of the Kruskal-Wallis test (p*-value) and Mann-Whitney U test with Holm's correction (p-value) are presented.

TABLE 21

Proportion of people with clinically relevant infection reported in Participant diary 1 (N = 123)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)	Placebo (N* = 24)
Gastrointestinal infection (%)	0/25 (0.0)	0/25 (0.0)	2/24 (8.3)
Respiratory tract infection (%)	1/25 (4.0)	3/25 (12.0)	6/24 (25.0)
Urinary tract infection (%)	1/25 (4.0)	2/25 (8.0)	1/24 (4.2)

N* = number of participants included in the ITT population

TABLE 22

Number of days with clinically relevant infection reported in Participant diary 1 (N = 123)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)	Placebo (N* = 24)
Gastrointestinal infection (p* = 0.081)	25	25	24
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.1 (0.4): 0 – 2

p-value vs placebo	0.434	0.434	/
Respiratory tract infection (p* = 0.212)	25	25	24
mean (SD): min – max	0.1 (0.6): 0 – 3	0.6 (2.2): 0 – 11	1.2 (2.4) 0 – 8
p-value vs placebo	0.142	0.236	/
Urinary tract infection (p* = 0.754)	25	25	24
mean (SD): min – max	0.1 (0.6): 0 – 3	0.2 (0.6): 0 – 2	0.2 (0.8): 0 – 4
p-value vs placebo	1.000	1.000	/

N* = number of participants included in the ITT population, NC = not calculable, *p* = p-value for Kruskal-Wallis test, p = p- value for Mann-Whitney U test.*

[0251] Kruskal-Wallis test did not show any significant differences in the number of days with clinically relevant infection treatment groups. However, a borderline statistically significant result was observed for clinically relevant gastrointestinal infection. This is probably due the fact that only no participants in the four probiotic treatment groups experienced clinically relevant gastrointestinal infection, while in probiotic group in total 2 days of such infection were observed, which could have happened by chance.

[0252] Nevertheless, compared to placebo, none of the study products containing probiotics showed a statistically significant difference.

[0253] Table 23 represents proportion of people who had reported at least one day with individual symptom of gastrointestinal infection in Participant diary 2. In Table 24 descriptive statistics of number of days with symptoms of gastrointestinal infection and results of the Kruskal-Wallis test (p*-value) and Mann- Whitney U test with Holm's correction (p-value) are presented.

TABLE 23

Proportion of people that had reported symptoms of gastrointestinal infection in Participant diary 2 (N = 118)

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)	Placebo (N* = 24)
Loss of appetite (%)	0/24 (0.0)	0/25 (0.0)	1/23 (4.3)
Diarrhea (%)	0/24 (0.0)	0/25 (0.0)	1/23 (4.3)
Constipation (%)	0/24 (0.0)	0/25 (0.0)	3/23 (13.0)
Vomiting (%)	0/24 (0.0)	0/25 (0.0)	1/23 (4.3)
Gases (%)	0/24 (0.0)	2/25 (8.0)	2/23 (8.7)
Bowel sounds (%)	0/24 (0.0)	0/25 (0.0)	0/23 (0.0)
Cramping/stomach pain (%)	1/24 (4.2)	1/25 (4.0)	2/23 (8.7)
Bloating (%)	1/24 (4.2)	0/25 (0.0)	1/23 (4.3)

N* = number of participants included in the ITT population

TABLE 24

Number of days with symptoms of gastrointestinal infection reported in Participant diary 2 (N = 118).

	Bacillus coagulans (N* = 25)	Probiotic cocktail (N* = 25)	Placebo (N* = 24)
Loss of appetite (p* = 0.389)	24	25	23
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.0 (0.2): 0 – 1
p-value vs placebo	0.921	1.000	/
Diarrhea (p* = 0.705)	24	25	23
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.0 (0.2): 0 – 1
p-value vs placebo	0.921	1.000	/
Constipation (p* = 0.013)	24	25	23
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.4 (1.5): 0 – 7
p-value vs placebo	0.212	0.260	/
Vomiting (p* = 0.389)	24	25	23
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.0 (0.2): 0 – 1
p-value vs placebo	0.921	1.000	/
Gases (p* = 0.660)	24	25	23

mean (SD): min – max	0.0 (NC): 0 – 0	0.1 (0.3): 0 – 1	0.3 (1.3): 0 – 6
p-value vs placebo	0.577	0.863	/
Bowel sounds (p* = 1.000)	24	25	23
mean (SD): min – max	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0	0.0 (NC): 0 – 0
p-value vs placebo	1.000	1.000	/
Cramping/stomach pain (p* = 0.705)	24	25	23
mean (SD): min – max	0.1 (0.6): 0 – 3	0.0 (0.2): 0 – 1	0.2 (0.9): 0 – 4
p-value vs placebo	1.000	1.000	/
Bloating (p* = 0.718)	24	25	23
mean (SD): min – max	0.1 (0.6): 0 – 3	0.0 (NC): 0 – 0	0.6 (2.7): 0 – 13
p-value vs placebo	1.000	1.000	/

N* = number of participants included in the ITT population, NC = not calculable, *p* = p-value for Kruskal-Wallis test, p = p- value for Mann-Whitney U test.*

[0001] A significant difference among groups was detected only in the number of days with constipation (p*= 0.013) probably due the fact that only three participants in Placebo group had reported this symptom in Participant diary 2, while in other four treatment groups none of the participants had reported this symptom. However, further analysis (*Mann-Whitney U test with Holm's correction*) where number of days with constipation was compared between individual probiotic group and placebo group did not show significant differences, probably due to low sample size.

[0002] Efficacy outcomes: Treatment period

[0003] No significant differences in gastrointestinal health of the participants at baseline among the treatment groups were detected. Participants were randomized to 5 treatment group therefore such results were expected.

[0004] Scores of items in Gut-brain axis questionnaire were lower at the end of the treatment period compared to baseline scores, meaning that the participants were less bothered by these symptoms at the end of the treatment period. This can be observed for all treatment groups including the placebo group.

[0005] No significant differences in change (end of treatment period minus baseline) of Gut-brain axis item scores among the treatment groups were detected. Compared to placebo group, none of the study products containing probiotics showed a statistically significant difference Gut-brain axis score change.

[0006] No significant differences in improvement of Gut-brain axis item scores among the treatment groups were detected. Compared to placebo group, none of the study products containing probiotics showed a statistically significant difference Gut-brain axis score improvement.

[0007] No significant differences in sum of scores of all items in Gut brain axis questionnaire among treatment groups were detected. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in the sum of Gut-brain axis items scores at baseline or at the end of treatment period.

[0008] No significant differences in cholesterol and triglyceride changes (absolute and relative change from baseline to the end of the treatment period) among treatment groups were detected. Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in HDL, LDL, TC or TG change (absolute and relative) from baseline to the end of treatment period. The same findings were observed in the PP population.

[0009] Cytokine (TNFα, IFNα, IFNβ, IFNγ, and IL6) levels at the end of the treatment period were below the limit of quantification (LOQ) in all treatment groups. For cytokine IL13 some values above LOQ were reported, but no statistically significant difference between the treatment groups was observed.

[0010] No significant changes in heavy metals (Cadmium, Lead, Mercury, Copper, Nickel, Zinc, and Arsenic) levels at the end of the treatment period compared to the baseline values were detected in the *Bacillus megaterium* group. The same findings were observed in the PP population.

[0011] Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in the number of days with symptoms of gastrointestinal infection. However, a borderline statistically significant result was observed for clinically relevant gastrointestinal infection. This is probably due the fact that only no participants in the four probiotic treatment groups experienced clinically relevant gastrointestinal infection, while in

probiotic group in total 2 days of such infection were observed, which could have happened by chance.

[0012] Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in the number of days with symptoms of respiratory tract infection. However, a statistically significant difference among treatment groups was detected in the number of days with runny nose - thick ($p = 0.018$). probably due the fact that only three participants in Probiotic cocktail group have reported this symptom, while in other four treatment groups none of the participants have reported this symptom.

[0013] However, further post hoc analysis (pairwise comparison vs. placebo) did not show any significant differences.

[0014] Compared to placebo, none of the study products containing probiotics showed a statistically significant difference in the number of days with symptoms of urinary infection.

[0015] No statistically significant differences were detected among the treatment groups in stool regularity.

[0016] A significant difference among groups was detected for stool consistency, that is in the proportion of loose stools in the total treatment period as well as in weeks 6 and 7 of the treatment period. However, further post hoc analysis (pairwise comparison vs. placebo) did not show any significant differences, probably due to the low sample size. The participants in the Probiotic cocktail group had the smallest proportion of loose stool per all stools.

[0017] Efficacy outcomes: Follow-up period

[0018] A significant difference among groups was detected only in the number of days with constipation ($p = 0.013$). However, further post hoc analysis (pairwise comparison vs. placebo) did not show any significant differences, probably due to low sample size.

[0019] None of the study products containing probiotics showed a statistically significant difference in the number of days with respiratory tract infection symptoms.

[0020] None of the study products containing probiotics showed a statistically significant difference in the number of days with urinary tract infection symptoms.

[0021] No significant differences were detected among the treatment groups in stool regularity and stool consistency.

[0022] Safety outcomes

[0023] In total 17 AEs were reported. Between 1 and 5 ADE were reported for each study product, most commonly gastroesophageal reflux (3 AEs), rash (2 AEs), and vertigo (2 AEs). No SAEs were reported.

[0024] Causality assessment revealed no relation between the reported AEs and the study products.

[0025] In summary, this study has addressed the safety and efficacy of new probiotics, namely *Bacillus coagulans*, *Bacillus clausii*, *Bacillus megaterium* and probiotic cocktail containing *Bacillus subtilis, Bacillus megaterium*, *Bacillus clausii* and *Bacillus coagulans*.

[0026] The gastrointestinal health of the participants at baseline among the treatment groups did not differ between the study groups, which was expected due to randomization.

[0027] The primary outcome of the study (safety) was achieved, as 17 AEs were reported in total with no SAEs. Causality assessment revealed no relation between the reported AEs and the study products.

[0028] None of the efficacy related outcomes showed any statistically significant difference, however this comes as no surprise due to small sample size per study group. Still, some trends favouring active products were observed, specifically in the Gut-brain axis scores and proportion of loose stools.

[0029] To conclude probiotic products showed to be safe to use in adults, and have shown some favourable trends regarding Gut-brain axis and stool consistency, however further studies with larger sample size, a run-in period to determine baseline stool consistency for each participant and use of validated questionnaires to determine the Gut-brain axis domains are needed in order to scientifically prove the efficacy of the studied products.

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[0082] The invention is not limited to the embodiment described herein but can be amended or modified without departing from the scope of the present invention.

[0083] The use of the terms "a," "an," "the," and similar referents in the context of describing the present invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Use of the term "about" is intended to describe values either above or below the stated value in a range of approximately ±10%; in other embodiments, the values may range in value above or below the stated value in a range of approximately ±5%; in other embodiments, the values may range in value above or below the stated value in a range of approximately ±2%; in other embodiments, the values may range in value above or below the stated value in a range of

approximately ±1%. The preceding ranges are intended to be made clear by context, and no further limitation is implied. All methods described herein can be performed in any suitable order unless otherwise indicated here in or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise stated. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0084] While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been put forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein can be varied considerably without departing from the basic principles of the invention.

[0085] All references cited herein are incorporated by reference in their entireties. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof, and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

CLAIMS:

1. A *Bacillus coagulans* strain comprising a purified microbial population that comprises one or more bacteria with a *gyrB* that shares at least 97% identity with SEQ ID NO: 1; and / or that comprises one or more bacteria with a 16S rRNA that shares at least 97% identity with SEQ ID NO: 2.

2. The *Bacillus coagulans* strain of Claim 1 that shares at least 97% identity with SEQ ID NO: 3.

3. The *Bacillus coagulans* strain of Claim 1 wherein the purified microbial population comprises a bacterium with a 16S nucleic acid sequence comprising SEQ ID NO:2.

4. The *Bacillus coagulans* strain of Claim 1 wherein the purified microbial population comprises a bacterium with *gyrB* nucleic acid sequence comprising SEQ ID NO: 1.

5. The *Bacillus coagulans* strain of Claim 1 wherein the purified microbial population comprises a bacterium with a 16S nucleic acid sequence comprising SEQ ID NO: 2 and with a *gyrB* nucleic acid sequence comprising SEQ ID NO: 1.

6. A microbial composition comprising the *Bacillus coagulans* strain of any one of Claims 1 to 5 together with a comestibly acceptable carrier and/or diluent.

7. The microbial composition of Claim 6, wherein a unit dose of the composition comprises 10^6-10^{13} CFU of the *Bacillus coagulans* strain.

8. The microbial composition of Claim 6 or 7, further comprising a mucous adherent excipient.

9. The microbial composition of any one of Claims 6 to 8, further comprising at least one further probiotic *Bacillus* strain.

10. The microbial composition of any one of Claims 6 to 9, wherein the microbial composition is formulated as a tablet, a pill, a capsule, a powder, a solution, a suspension, or an emulsion.

11. The microbial composition of any one of Claims 6 to 9, wherein the microbial composition is formulated as a food.

12. The *Bacillus coagulans* strain of any one of Claims 1 to 5, for use in preventing or treating vaginal infections, urinary tract infections, gastrointestinal infections, gastrointestinal diseases, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health.

13. A method of preventing or treating vaginal infections, urinary tract infections, gastrointestinal infections, gastrointestinal diseases, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health, the method comprising administering the *Bacillus coagulans* strain of any one of Claims 1 to 5.

14. The microbial composition of any one of Claims 6 to 11, for use in preventing or treating vaginal infections, urinary tract infections, gastrointestinal infections, gastrointestinal diseases, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health.

15. A method of preventing or treating vaginal infections, urinary tract infections, gastrointestinal infections, gastrointestinal diseases, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health and cardiovascular health, the method comprising administering the microbial composition of any one of Claims 6 to 11.

16. A method of improving microbiome within a subject, comprising administering to the subject a composition comprising a probiotic, wherein the probiotic comprises the *Bacillus coagulans* strain of any one of Claims 1 to 5.

17. The *Bacillus coagulans* strain of any one of Claims 1 to 5 for use as a probiotic, wherein optionally the bacterial strain(s) is(are) associated with acceptable carrier or delivery vehicle(s) and optionally adjuvant component(s) within a single composition, or separate compositions comprising a mixture of distinct bacterial strains.

18. Use of a *Bacillus coagulans* strain as in any one of Claims 1 to 5 in the manufacture of a medicament for the treatment of vaginal infections, urinary tract infections, gastrointestinal infections, gastrointestinal diseases, improving immune health, protection against oxidative stress, cleansing and detoxification, metabolic health, cardiovascular health, and/or skin health.

Phylogenetic tree (16S) of *Bacillus spp.* arranged in clades.

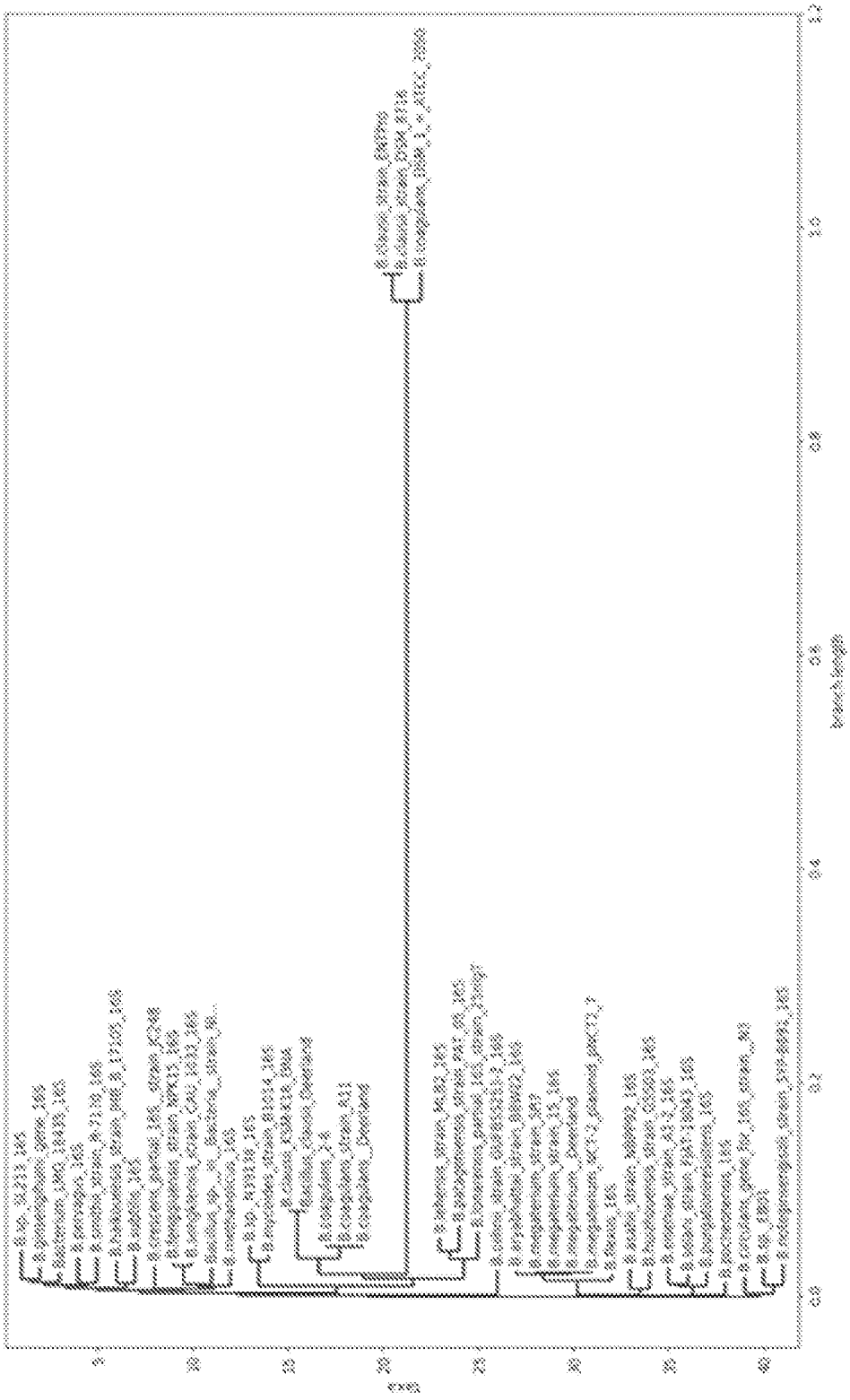

FIG. 1

Phylogenetic tree (*gyrB*) of *Bacillus spp.* arranged in clades.

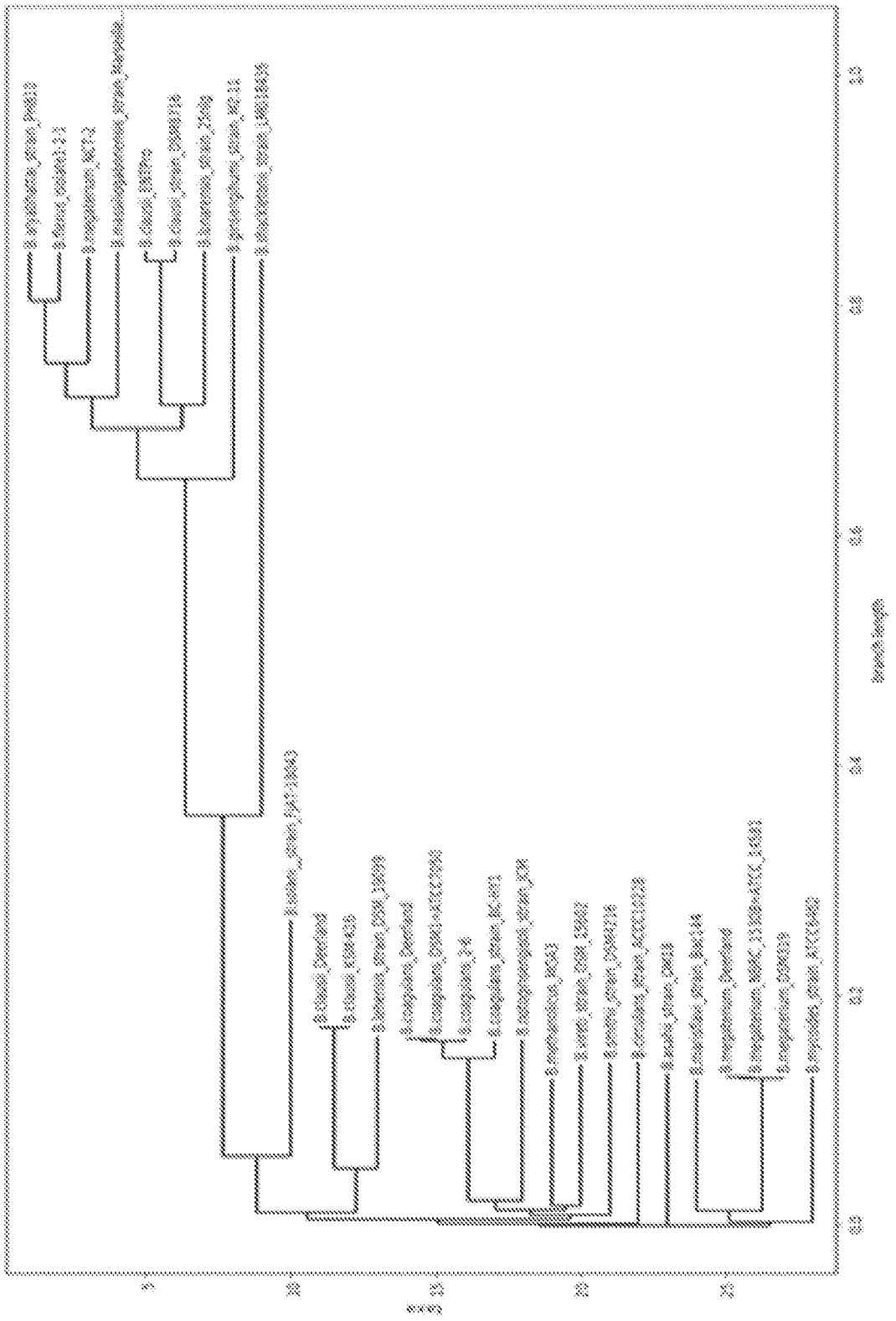

FIG. 2

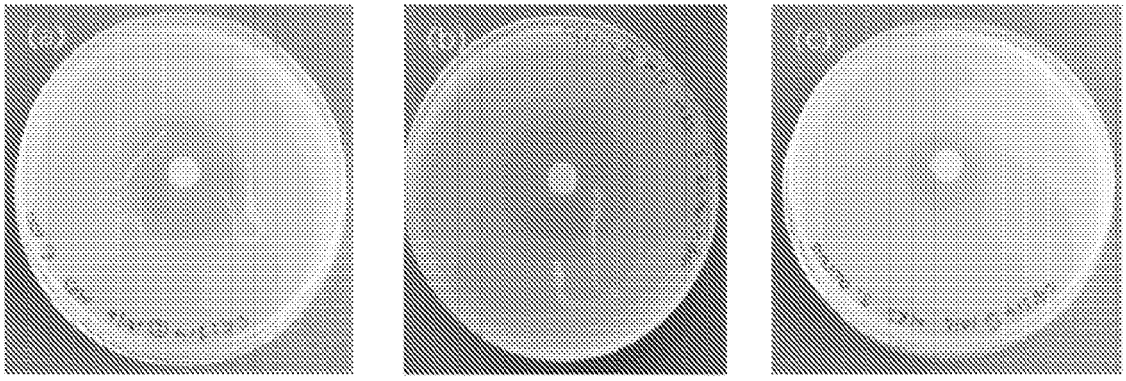

FIG. 3

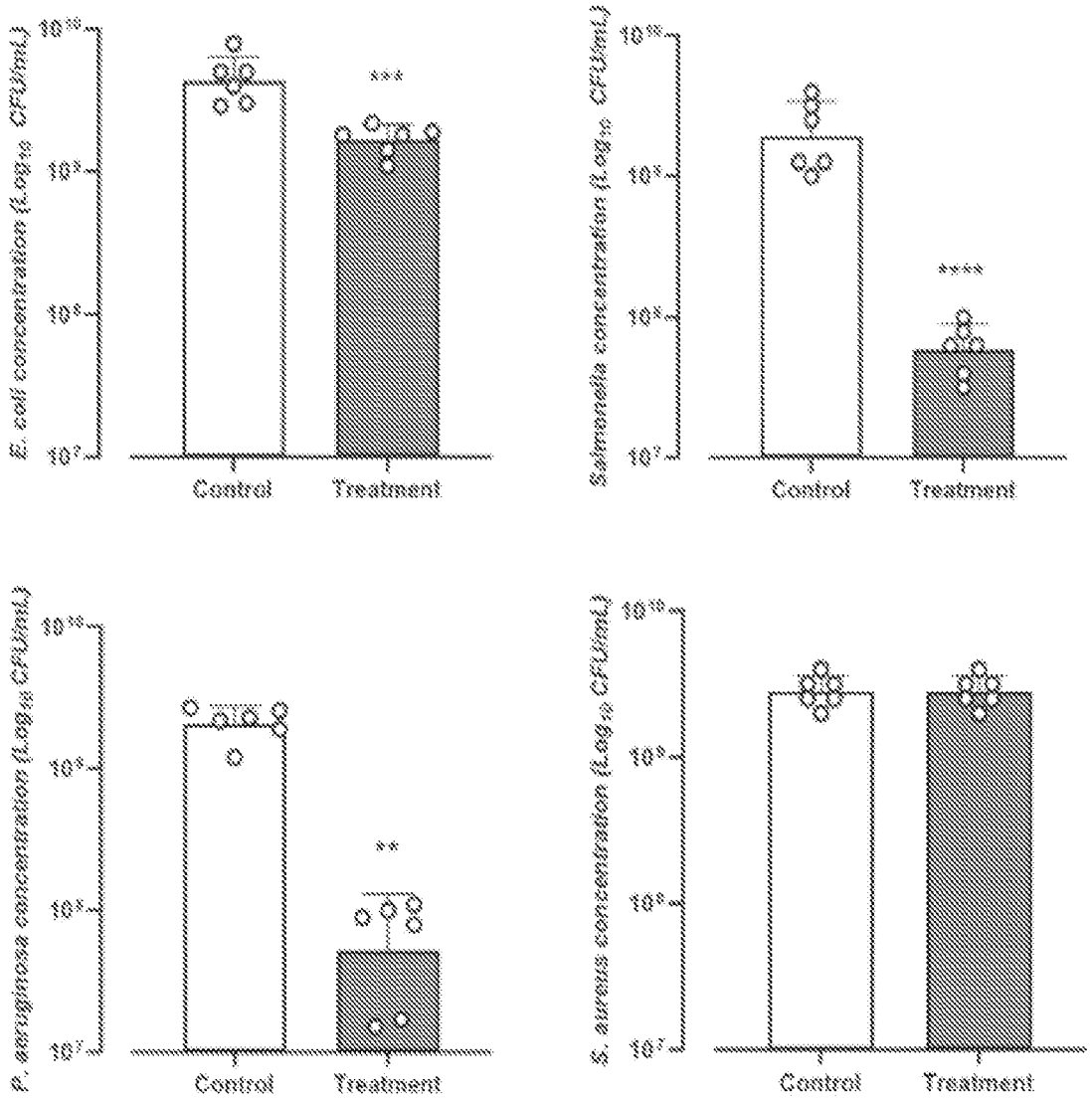
E. coli concentration (Log10 CFU/mL)
Salmonella concentration (Log10 CFU/mL)
P. aeruginosa concentration (Log10 CFU/mL)
S. aureus concentration (Log10 CFU/mL)
Control
Treatment
***
****
**

FIG. 4

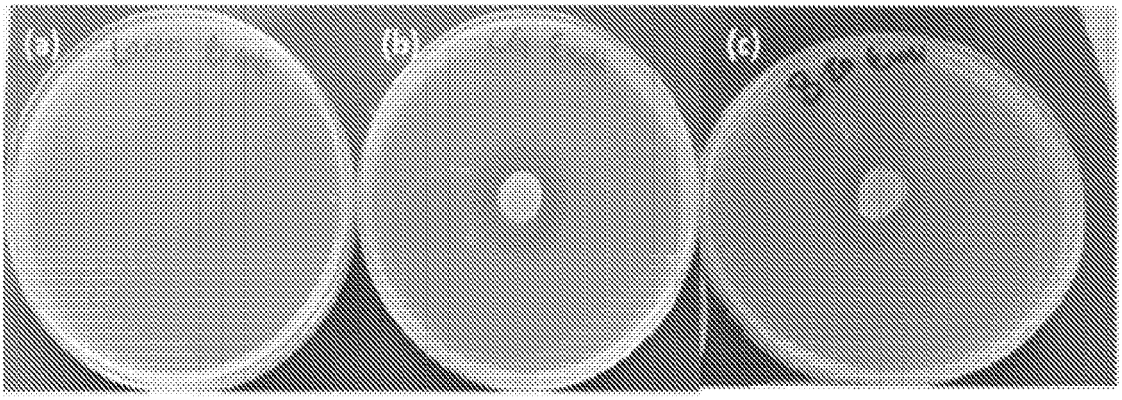

FIG. 5

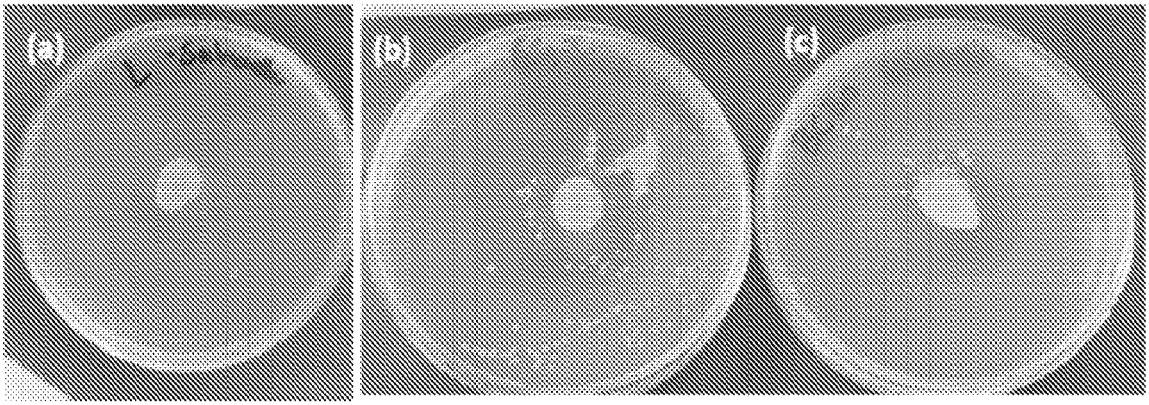

FIG. 6

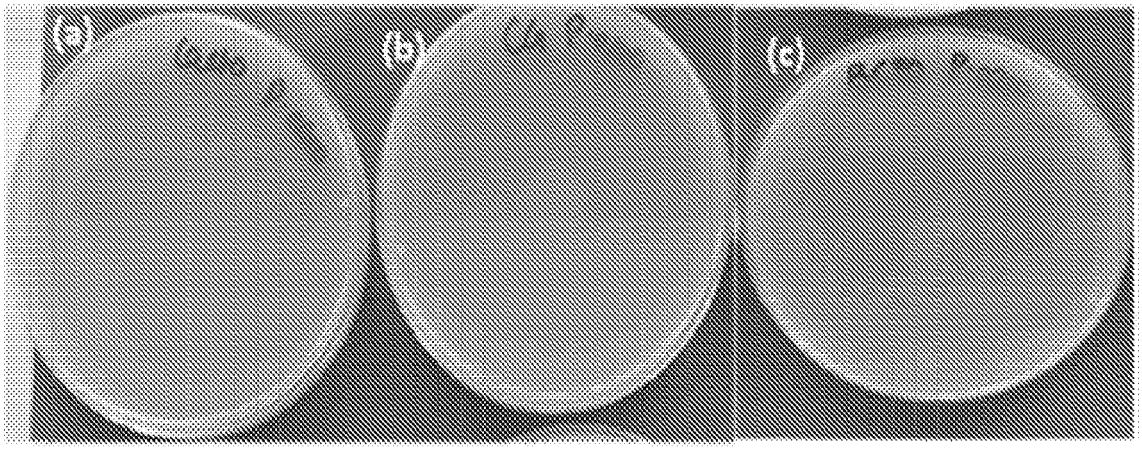

FIG. 7

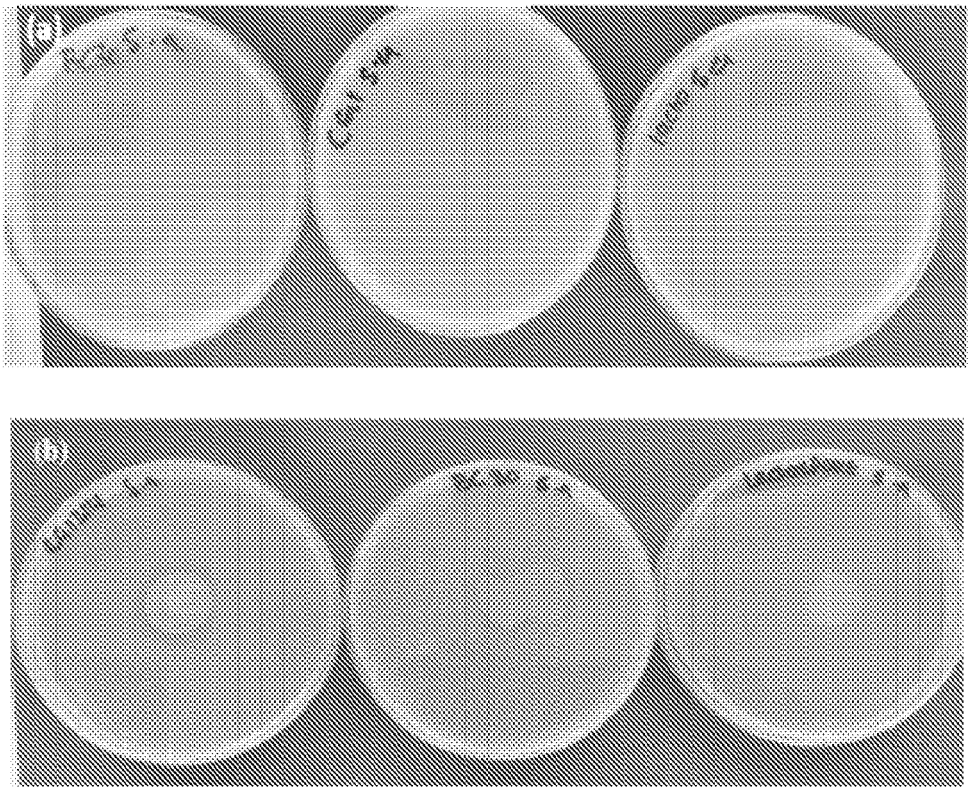

FIG. 8

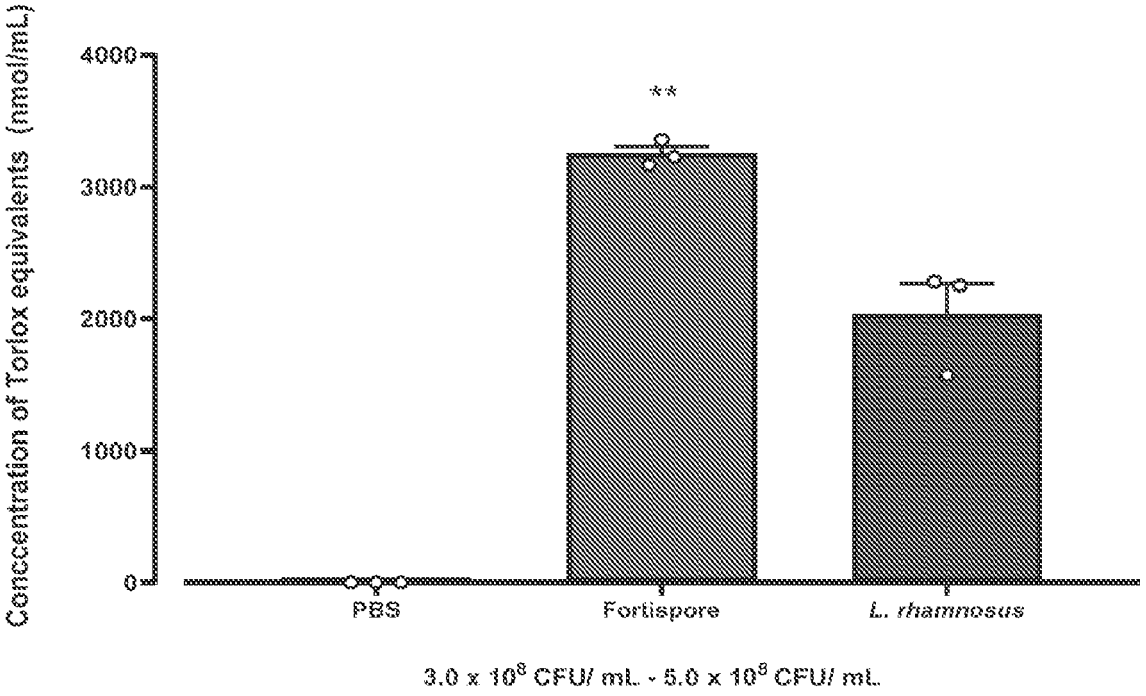
Concentration of Torlox equivalents (nmol/mL)
4000
3000
2000
1000
0
**
PBS
Fortispore
L. rhamnosus
3.0 x 10^8 CFU/ mL - 5.0 x 10^8 CFU/ mL

FIG. 9

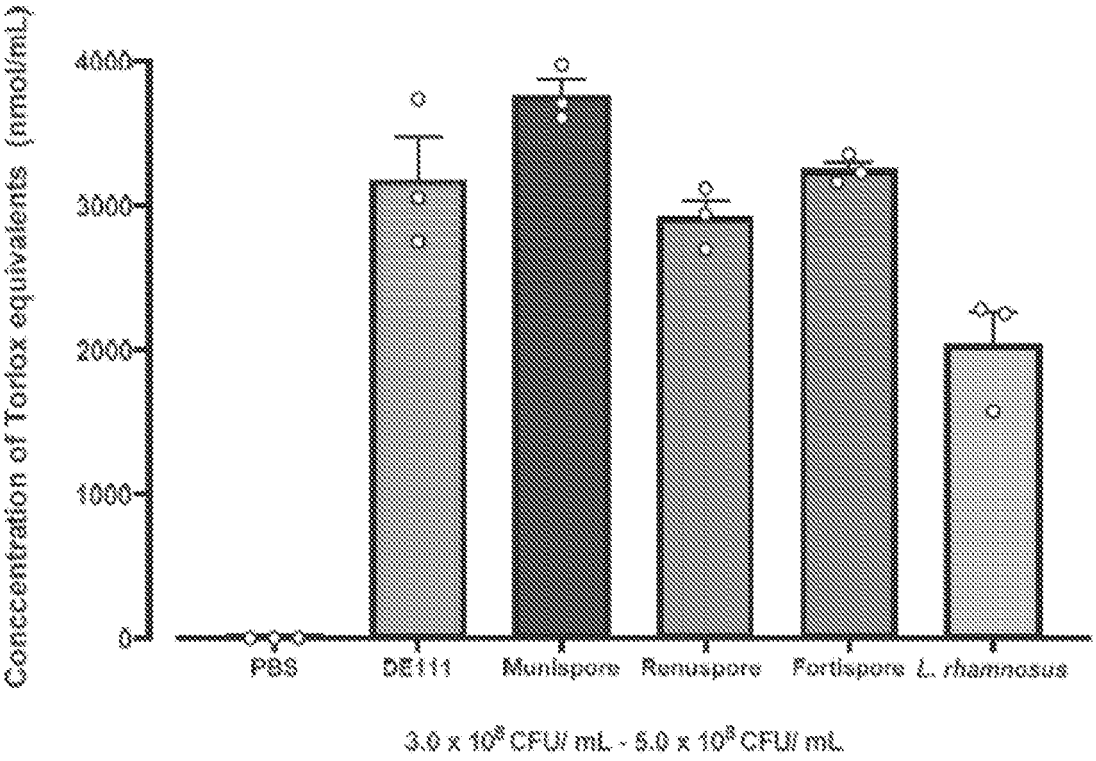
Concentration of Torlox equivalents (nmol/mL)
4000
3000
2000
1000
0
PBS
DE111
Munispore
Renuspore
Fortispore
L. rhamnosus
3.0 x 10^8 CFU/ mL - 5.0 x 10^8 CFU/ mL

FIG. 10

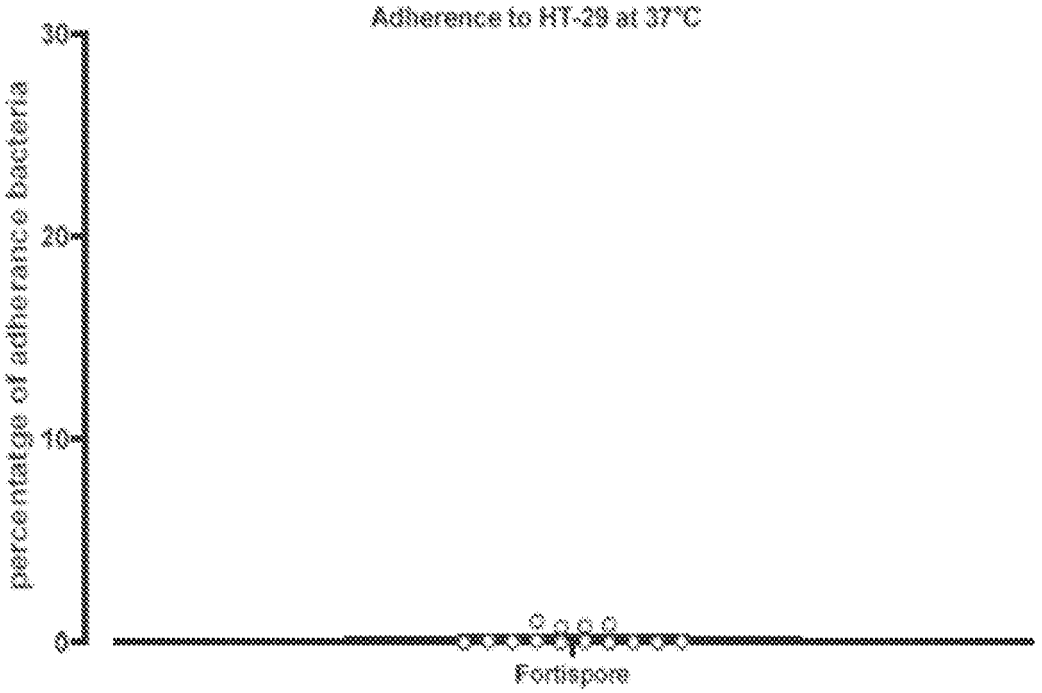
Adherence to HT-29 at 37°C
percentage of adherance bacteria
30
20
10
0
Fortispore

FIG. 11

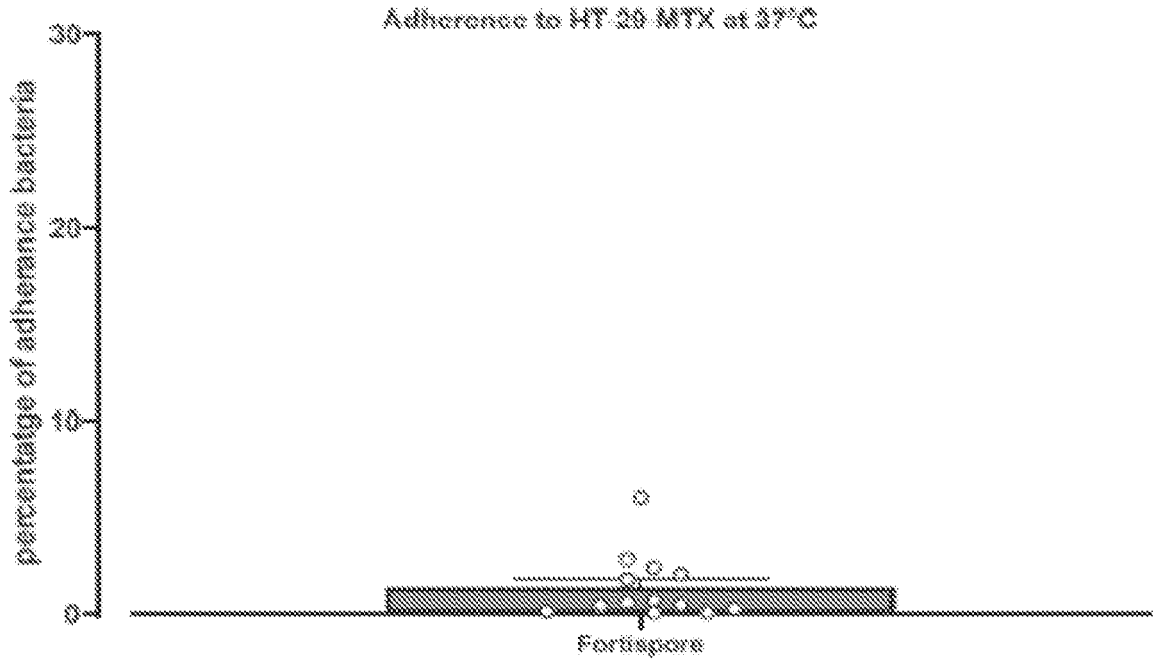
Adherence to HT-29 MTX at 37°C
percentage of adherance bacteria
30
20
10
0
Fortispore

FIG. 12

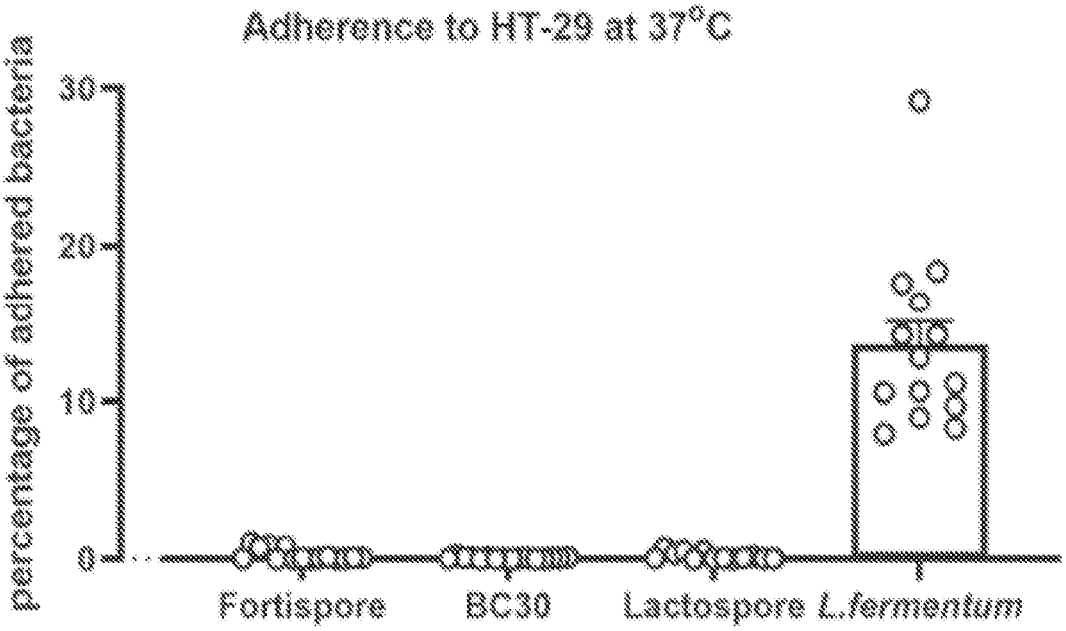
Adherence to HT-29 at 37°C
percentage of adhered bacteria
30
20
10
0
Fortispore
BC30
Lactospore
L.fermentum

FIG. 13

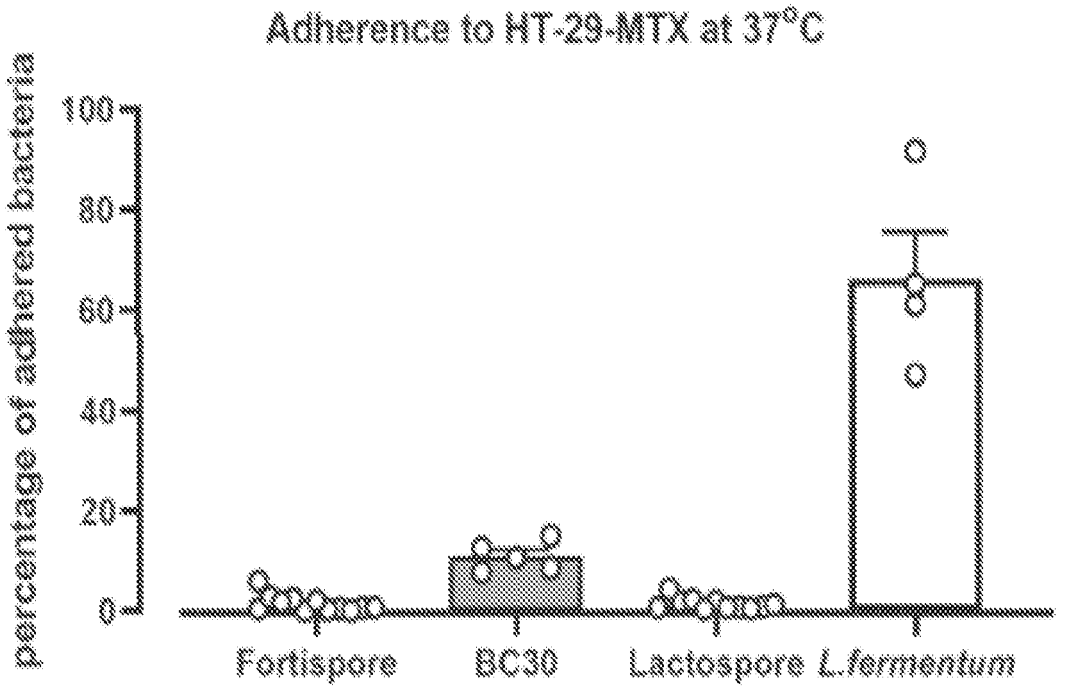
Adherence to HT-29-MTX at 37°C
percentage of adhered bacteria
100
80
60
40
20
0
Fortispore
BC30
Lactospore
L.fermentum

FIG. 14

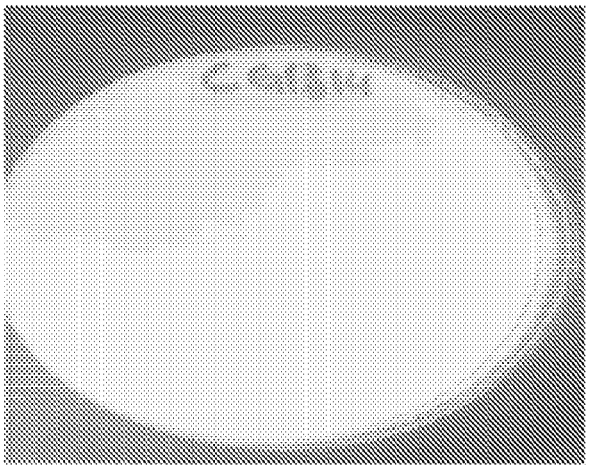

FIG. 15

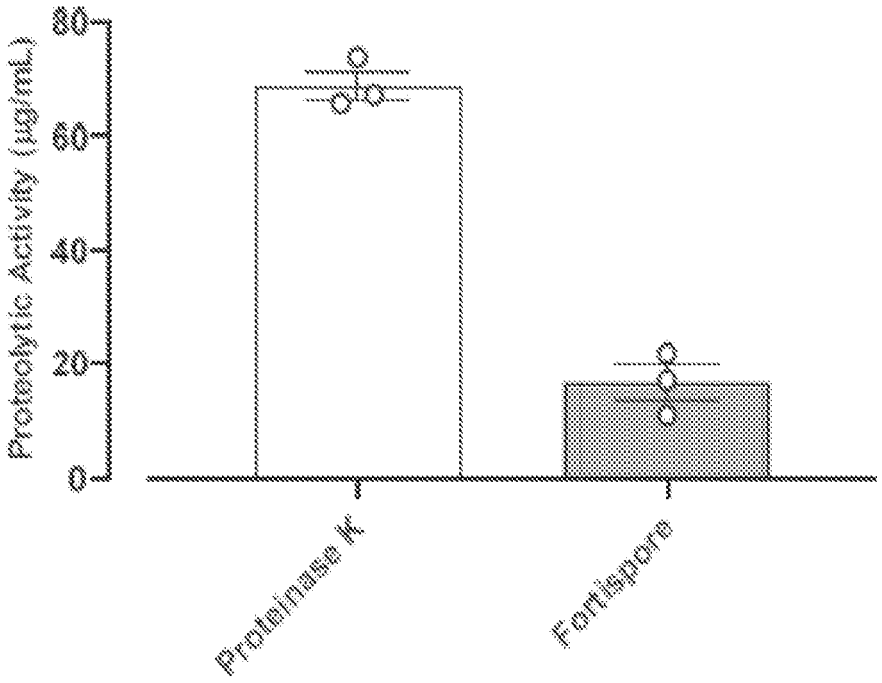
Proteolytic Activity (µg/mL)
80
60
40
20
0
Proteinase K
Fertispore

FIG. 16

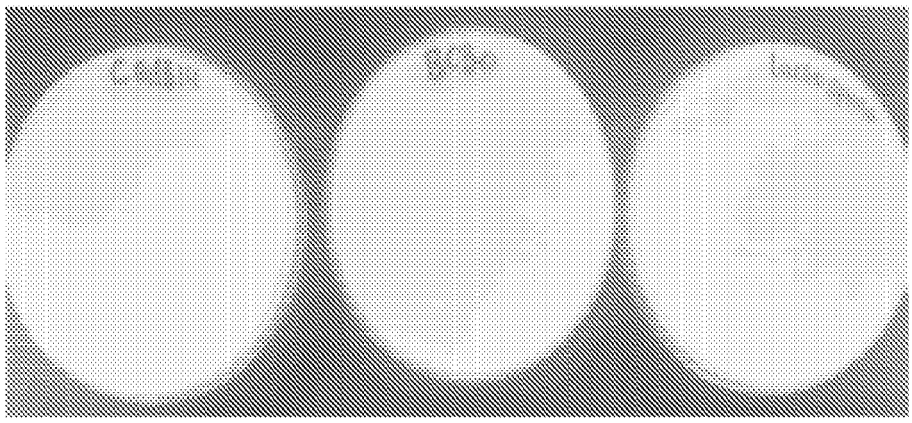

FIG. 17

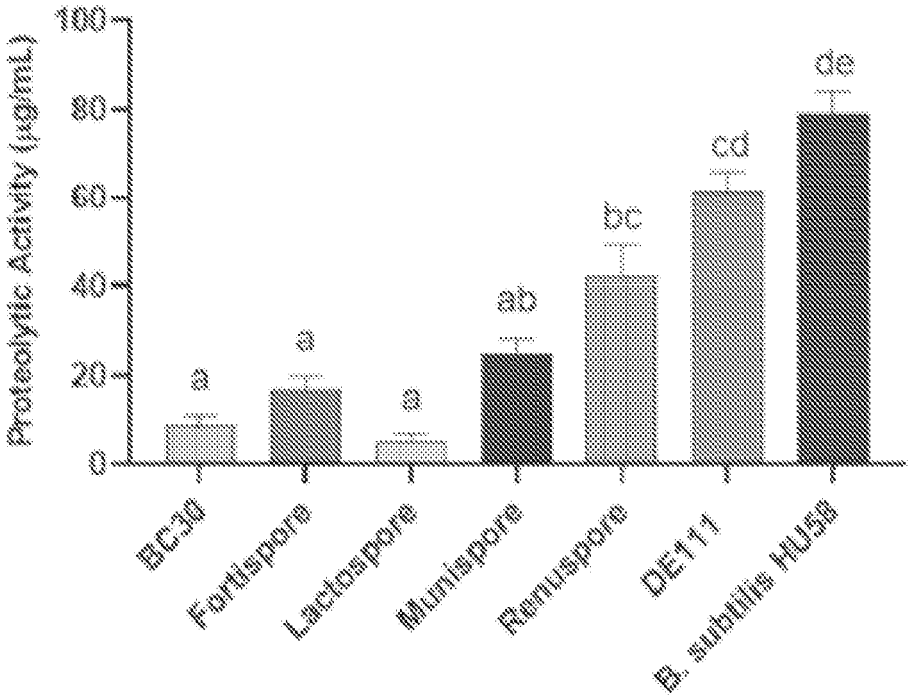
Proteolytic Activity (µg/mL)
100
80
60
40
20
0
a
a
a
ab
bc
cd
de
BC30
Fortispore
Lactospore
Munispore
Renuspore
DE111
B. subtilis HU58

FIG. 18

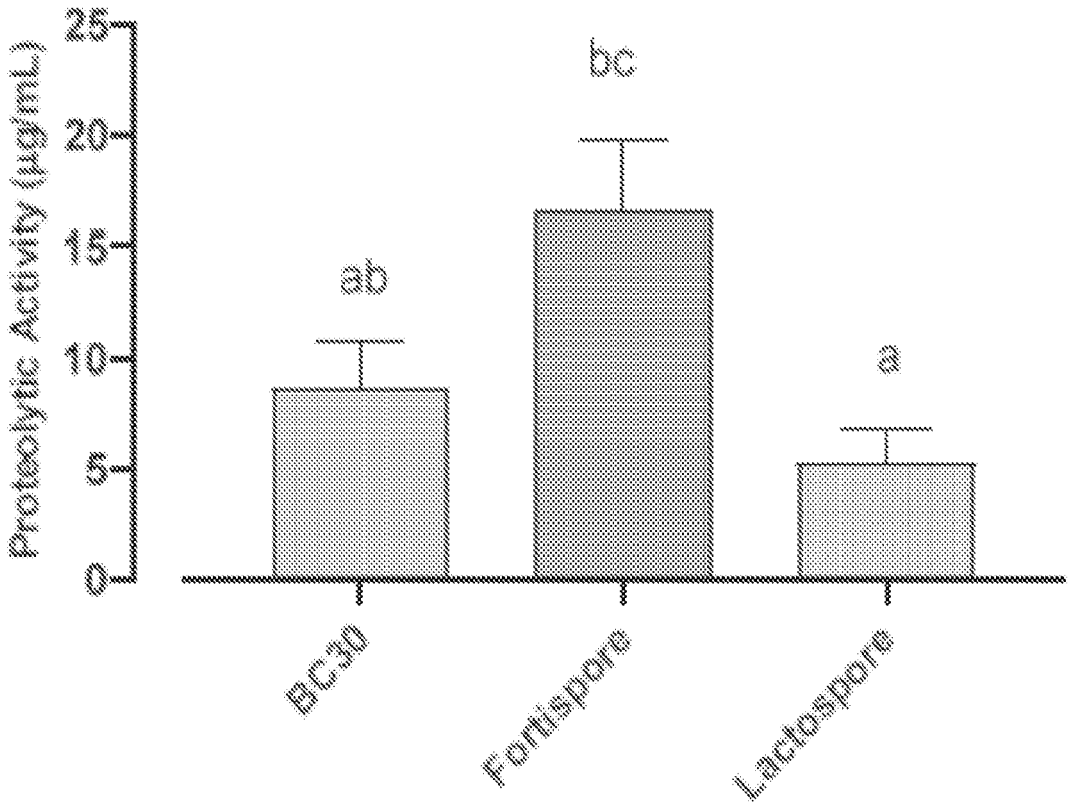
Proteolytic Activity (µg/mL)
25
20
15
10
5
0
ab
bc
a
BC30
Fortispore
Lactospore

FIG. 19

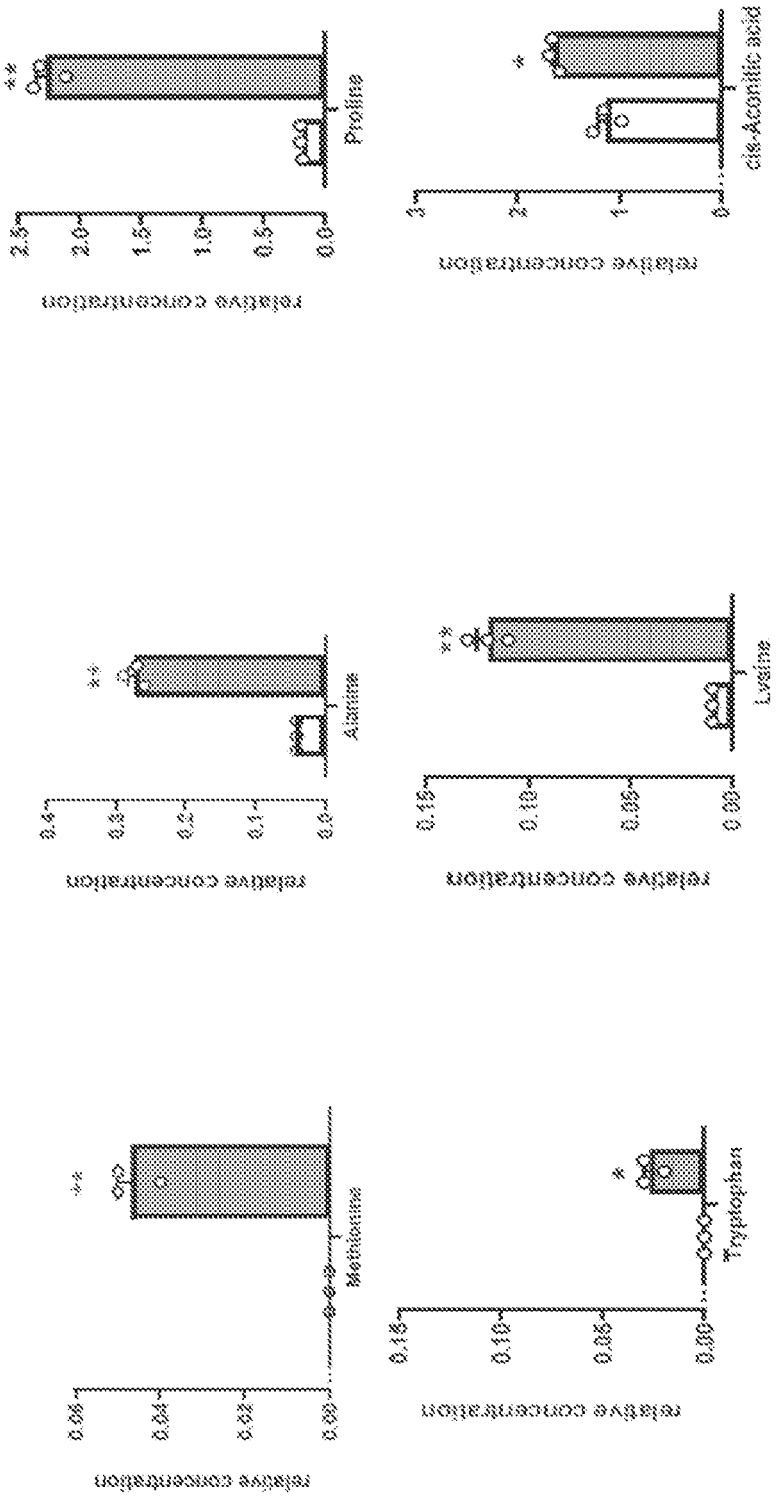
Proline
relative concentration
2.5
2.0
1.5
1.0
0.5
0.0
cis-Aconitic acid
relative concentration
3
2
1
0
Alanine
relative concentration
0.4
0.3
0.2
0.1
0.0
Lysine
relative concentration
0.15
0.10
0.05
0.00
Methionine
relative concentration
0.06
0.04
0.02
0.00
Tryptophan
relative concentration
0.15
0.10
0.05
0.00

FIG. 20

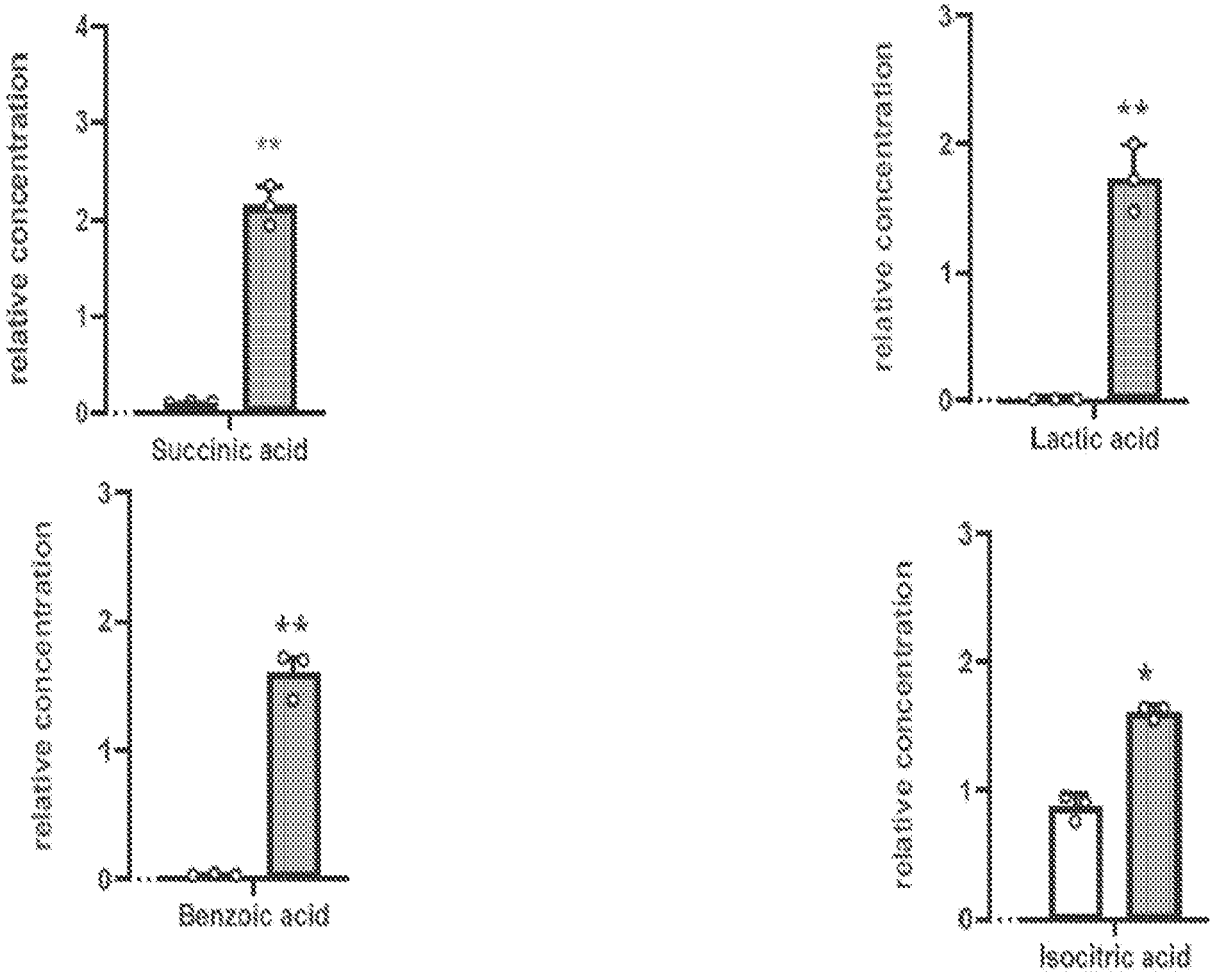
relative concentration
Succinic acid
Lactic acid
Benzoic acid
Isocitric acid

FIG. 21

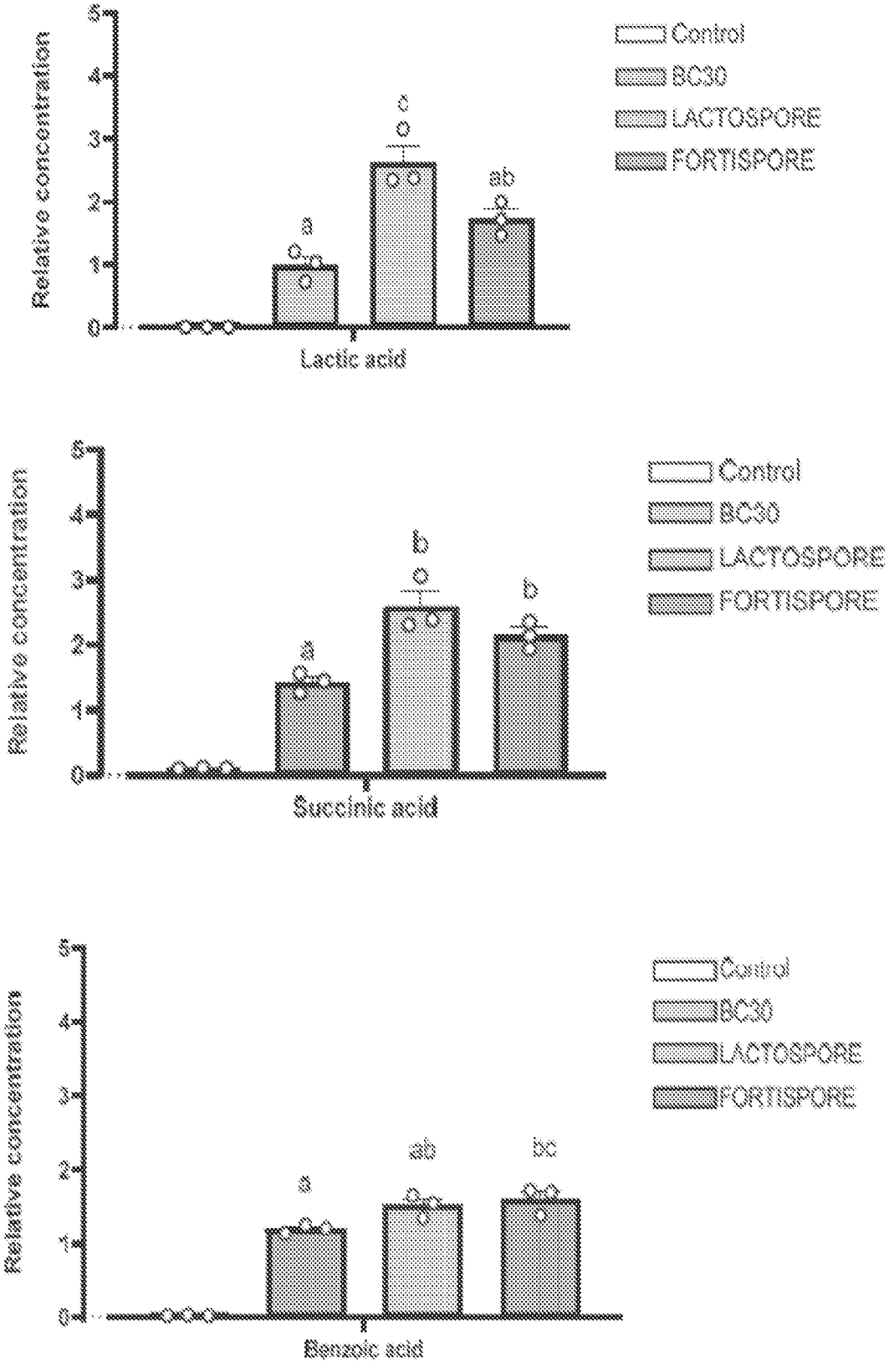
Relative concentration
0
1
2
3
4
5
a
c
ab
Lactic acid
Control
BC30
LACTOSPORE
FORTISPORE
Relative concentration
a
b
b
Succinic acid
Control
BC30
LACTOSPORE
FORTISPORE
Relative concentration
a
ab
bc
Benzoic acid
Control
BC30
LACTOSPORE
FORTISPORE

FIG. 22

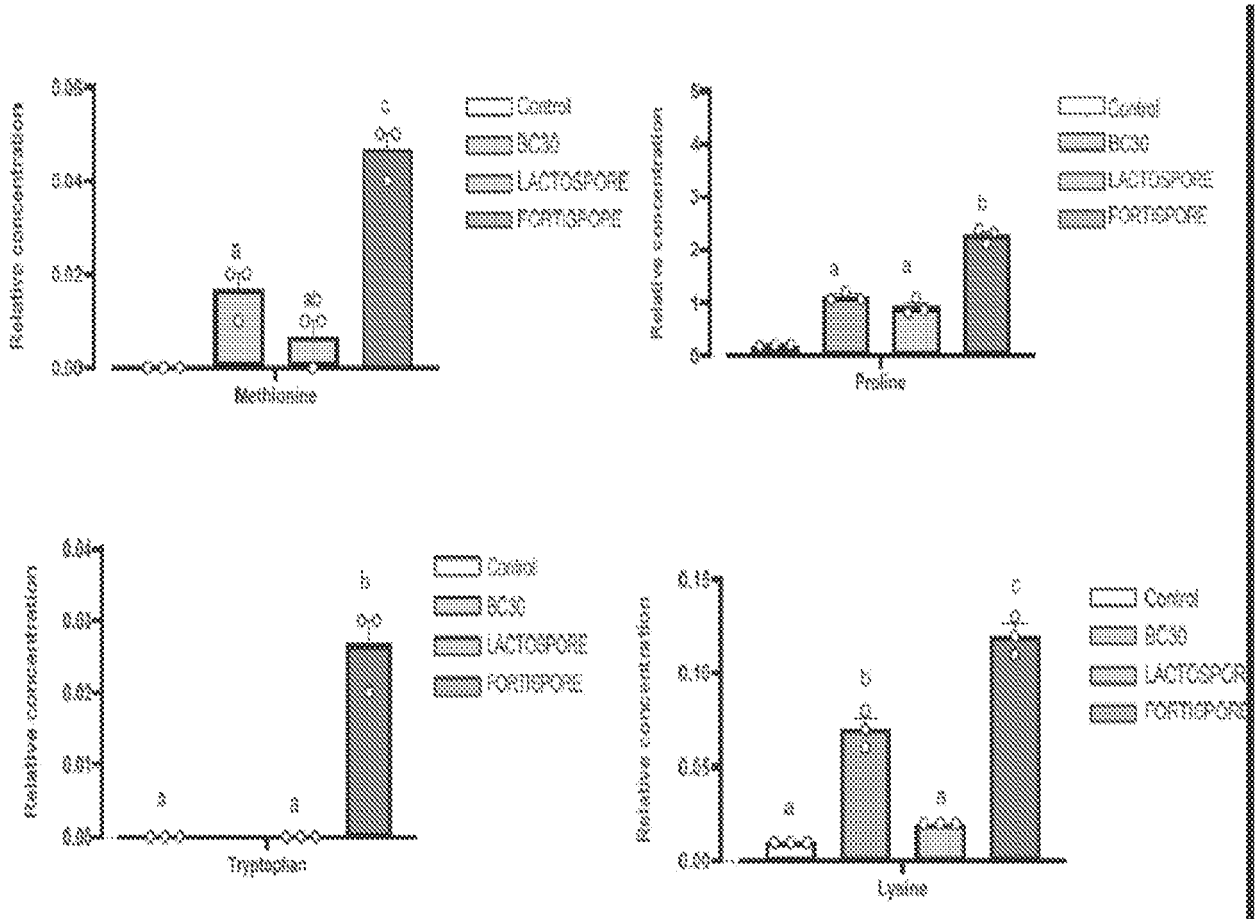
Relative concentration
Control
BC30
LACTOSPORE
FORTISPORE
Methionine
Proline
Tryptophan
Lysine

FIG. 23

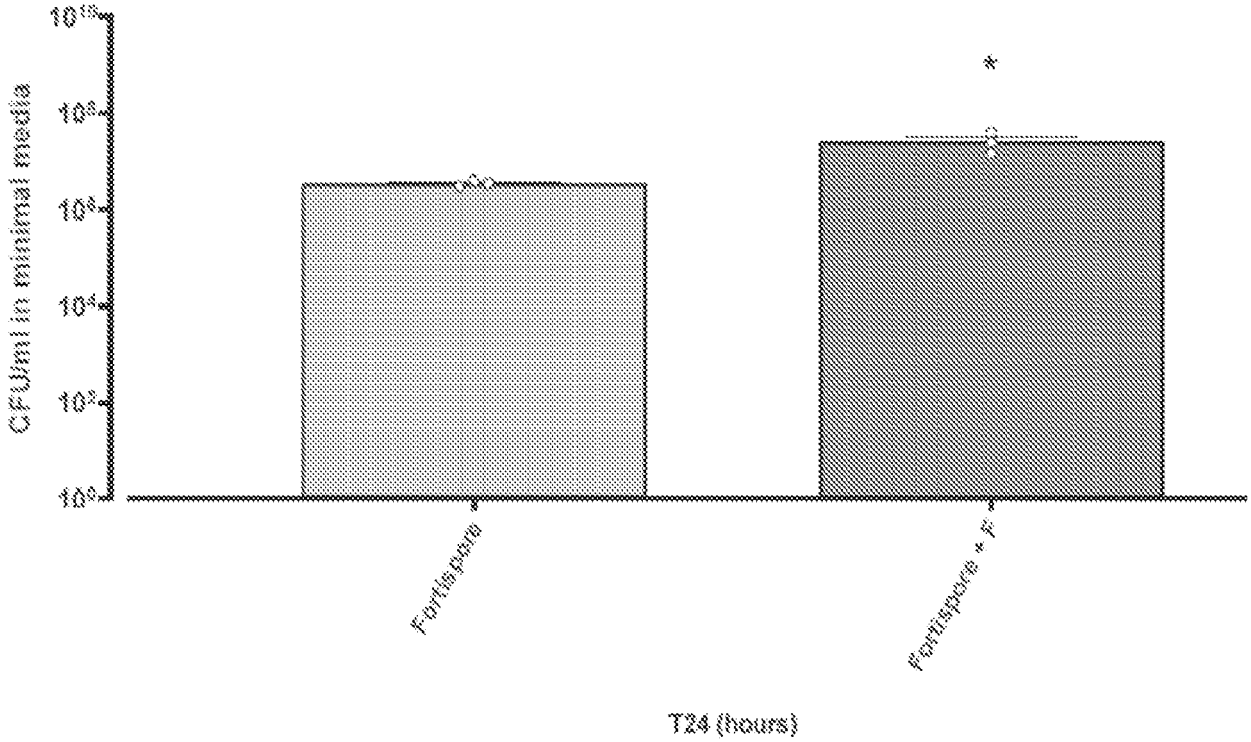

FIG. 24

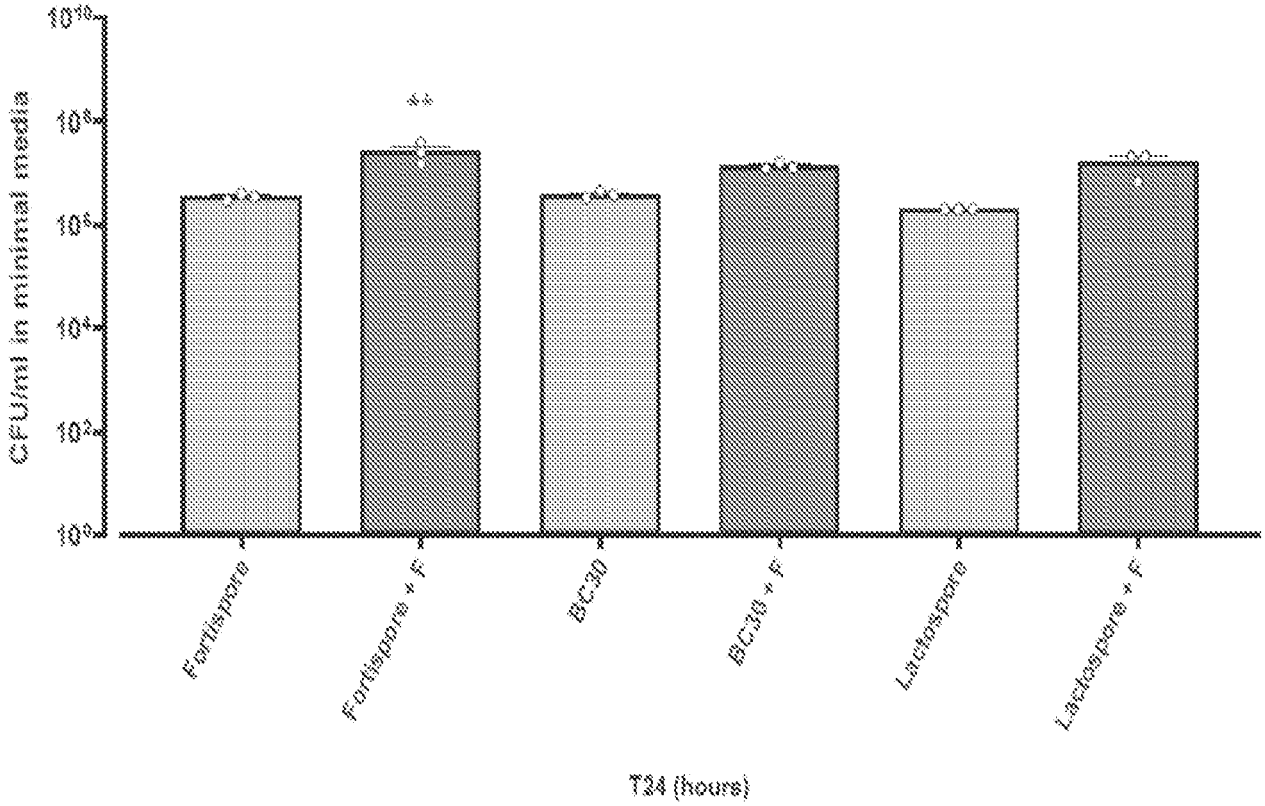
CFU/ml in minimal media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
**
Fortispore
Fortispore + F
BC30
BC30 + F
Lactospore
Lactospore + F
T24 (hours)

FIG. 25

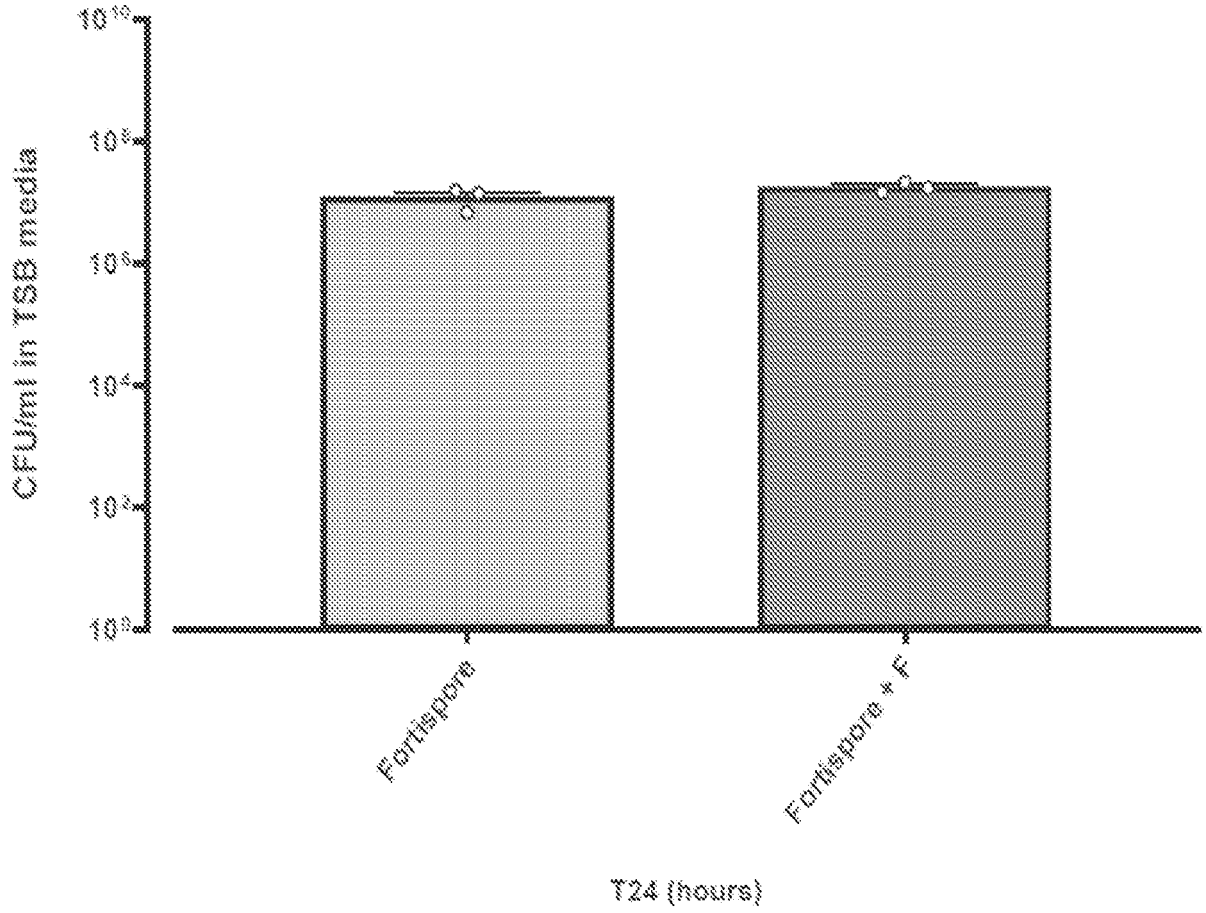

FIG. 26

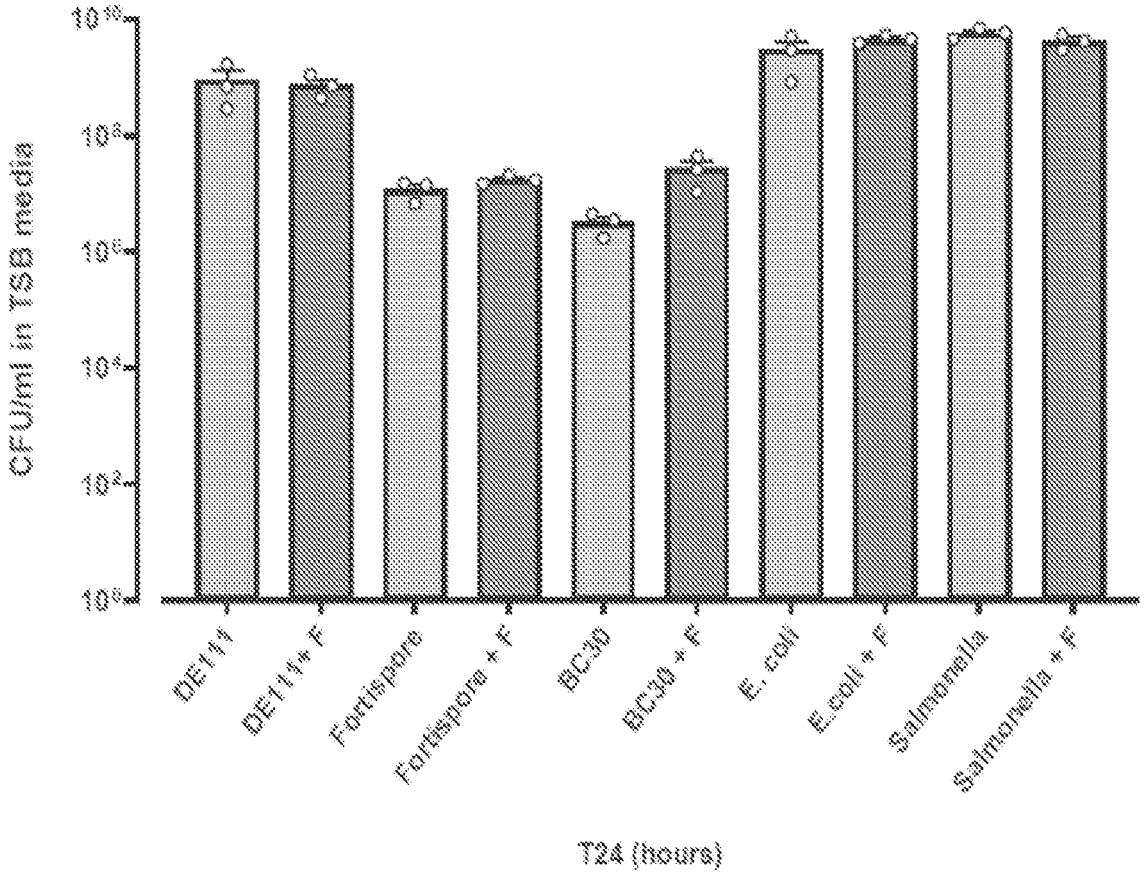
CFU/ml in TSB media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
DE111
DE111 + F
Fortispore
Fortispore + F
BC30
BC30 + F
E. coli
E. coli + F
Salmonella
Salmonella + F
T24 (hours)

FIG. 27

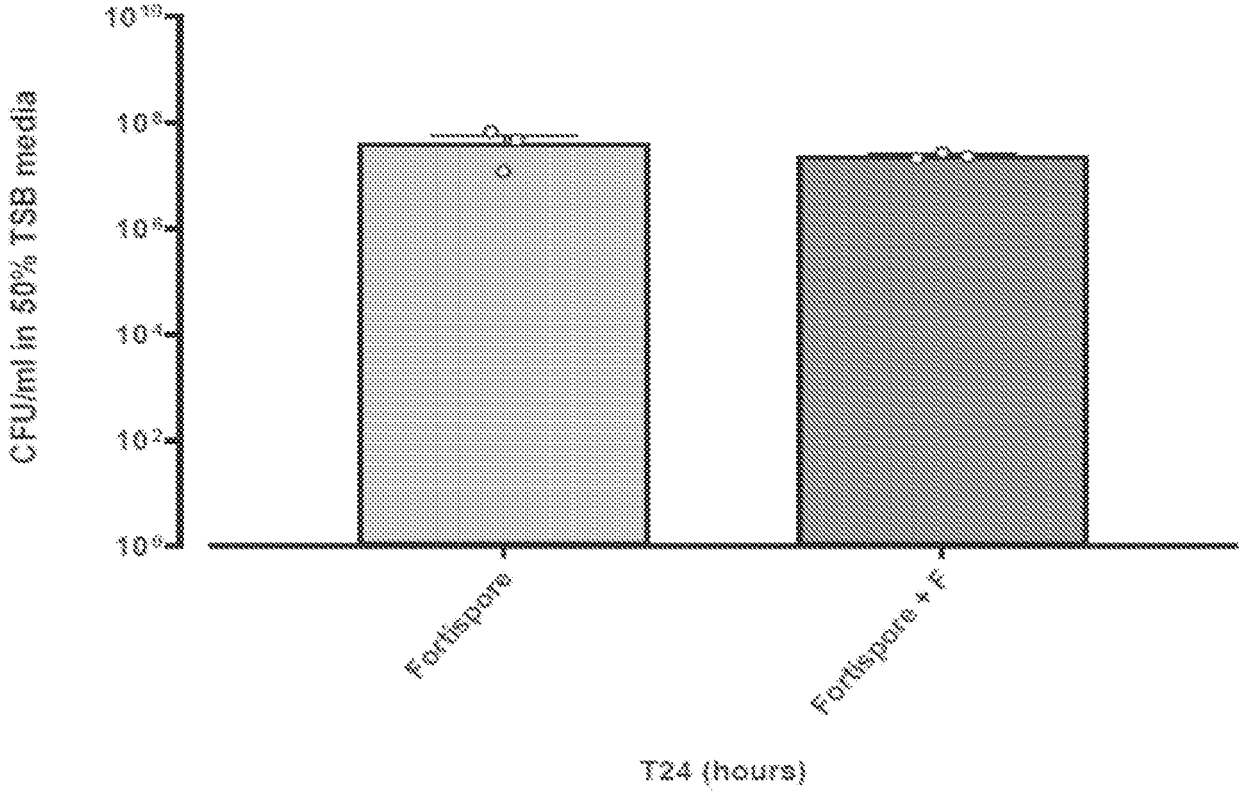
CFU/ml in 50% TSB media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
Fortispore
Fortispore + F
T24 (hours)

FIG. 28

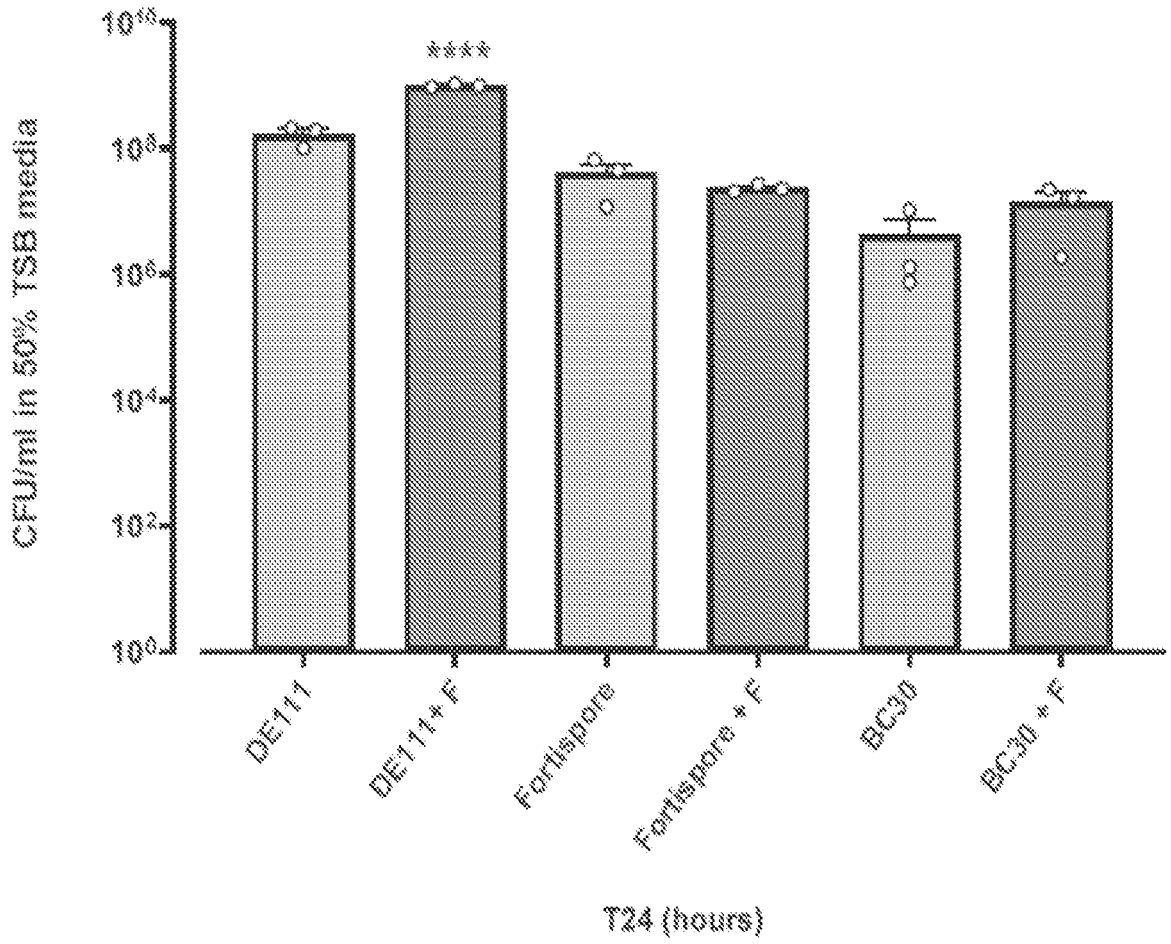
CFU/ml in 50% TSB media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
****
DE111
DE111 + F
Fortispore
Fortispore + F
BC30
BC30 + F
T24 (hours)

FIG. 29

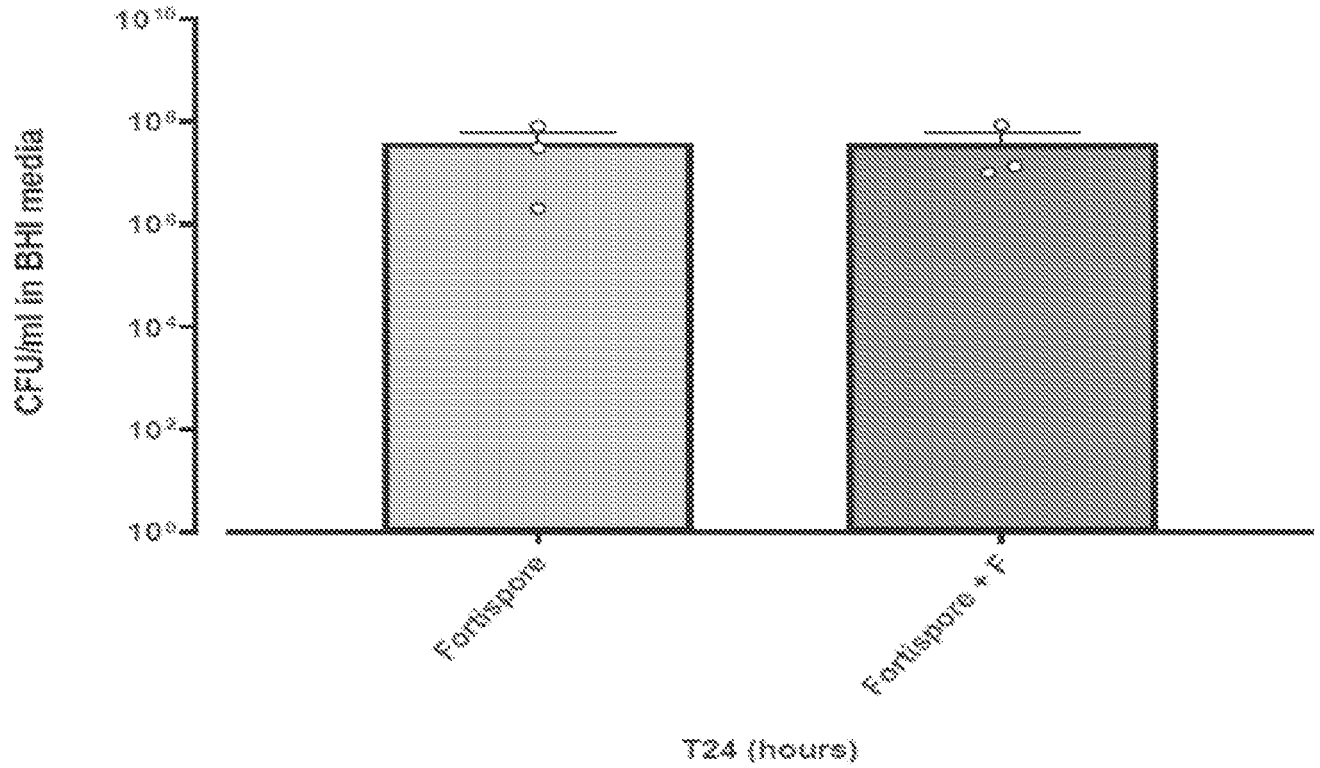
CFU/ml in BHI media
10^10
10^8
10^6
10^4
10^2
10^0
Fortispore
Fortispore + F
T24 (hours)

FIG. 30

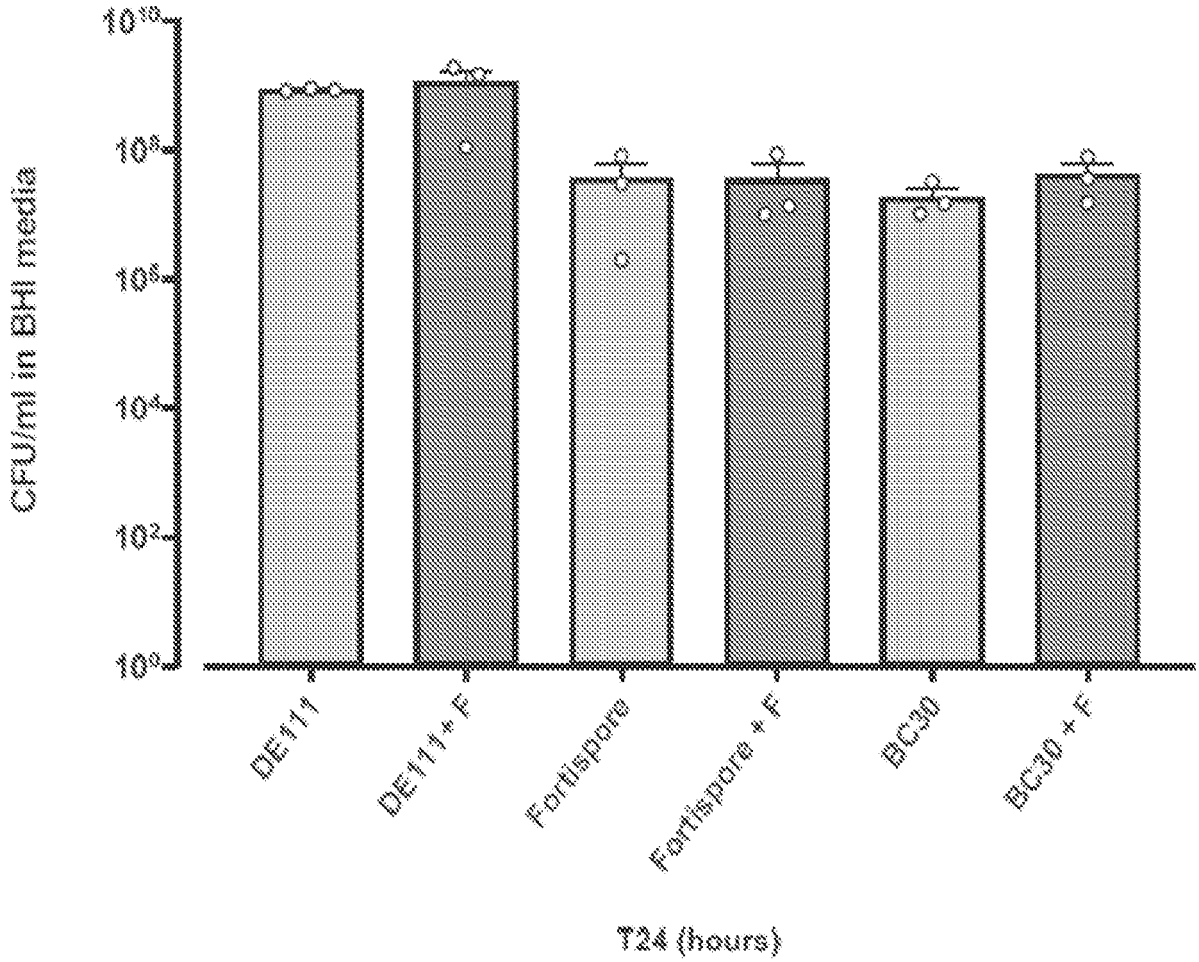
CFU/ml in BHI media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
DE111
DE111 + F
Fortispore
Fortispore + F
BC30
BC30 + F
T24 (hours)

FIG. 31

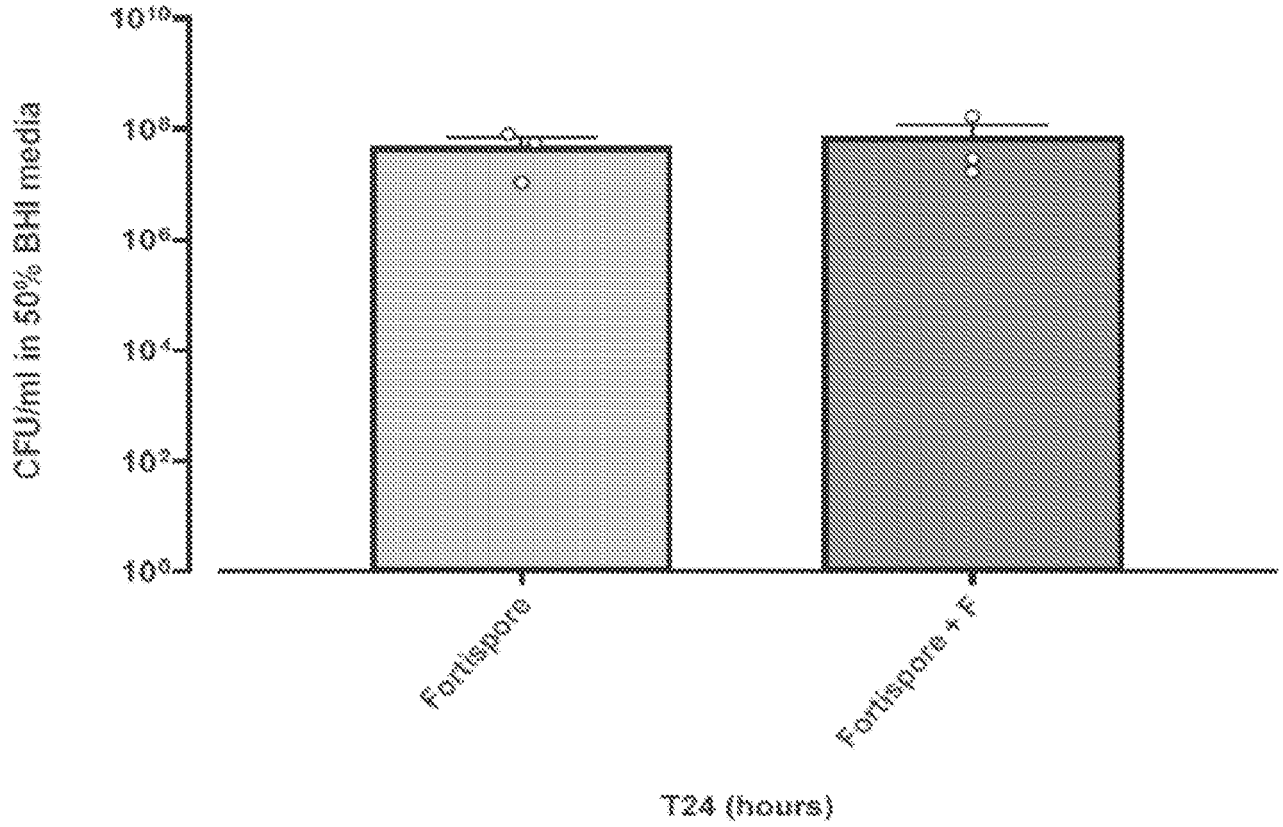

FIG. 32

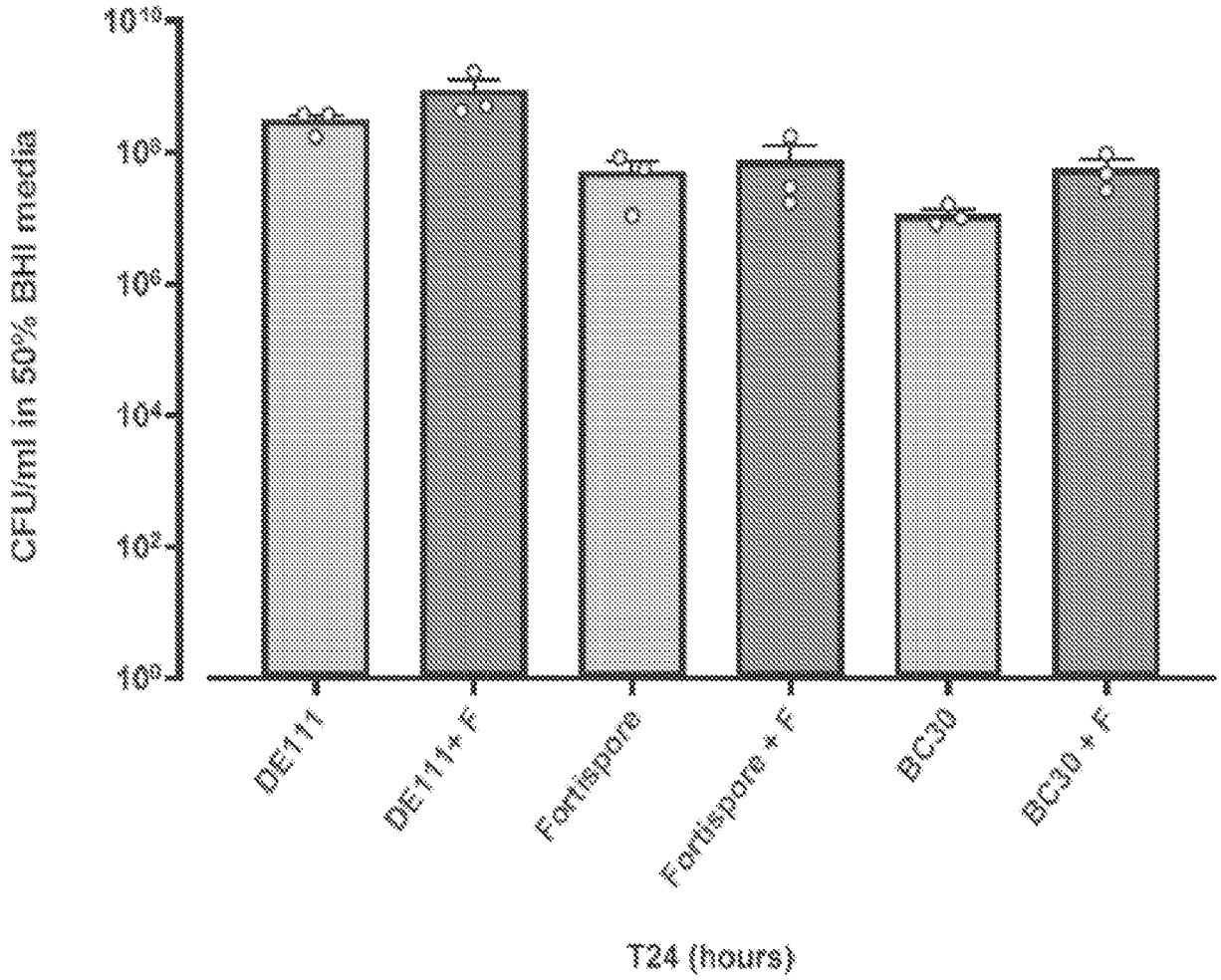
CFU/ml in 50% BHI media
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
DE111
DE111+F
Fortispore
Fortispore + F
BC30
BC30 + F
T24 (hours)

FIG. 33