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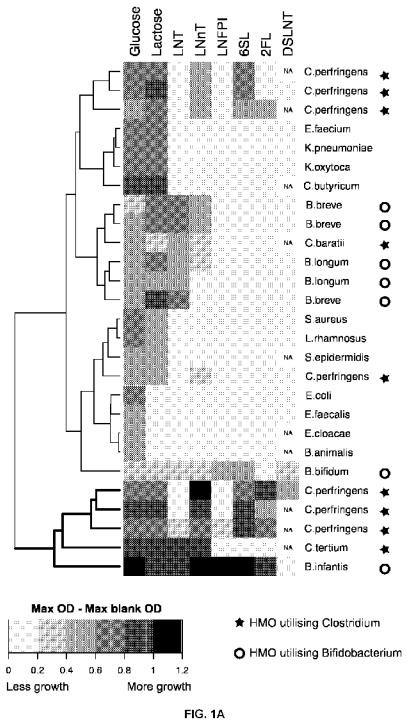


FIG. 1A

(57) Abstract: The present invention provides a probiotic composition for improving the gut microbiome and/or gut health of a subject comprising at least one Clostridium species selected from *Clostridium perfringens, Clostridium baratii, Clostridium tertium and Clostridium paraputrificum*. It also provides postbiotic compositions from these Clostridium species for the same purpose. Products comprising the compositions and methods of using the compositions are also provided herein.

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Probiotic and postbiotic compositions, products and uses thereof

The present invention provides a probiotic composition for improving the gut microbiome and/or gut health of a subject comprising at least one *Clostridium* species selected from *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*. It also provides postbiotic compositions from these *Clostridium* species for the same purpose. Products comprising the compositions and methods of using the compositions are also provided herein.

Background

The bacteria and other microorganisms that colonise the gut, termed the gut microbiome, play important roles in health and disease. This is especially true during the first year of life, where the gut microbiome contributes to education and training of the immune system, which can have implications for long term health.

Receipt of breast milk is one of the most important modulators of the infant gut microbiome, due in part to the direct provision of prebiotics. Prebiotics are non-digestible substrates that promote the growth of beneficial bacteria, termed probiotics, and an example of prebiotics in breast milk is human milk oligosaccharides (HMOs). HMOs are abundant unconjugated sugars that reach the infant gut intact where they are consumed by selected probiotic bacteria.

Most work to date has focused on species within the *Bifidobacterium* genus, due in part to the well described genetic capability of *Bifidobacterium* species to use HMOs. One proposed mechanism involves selected *Bifidobacterium* species consuming HMOs as an energy source and releasing short chain fatty acids (SCFAs) into the gut lumen. These SCFAs may promote health through a myriad of ways including providing an important energy source for epithelial cells, improving barrier integrity, stimulating goblet cells to produce mucus, anti-inflammatory properties, and protecting against infection.

Unlike infants born at term, preterm infants born under 32 weeks gestation typically spend the initial weeks or months of life in the neonatal intensive care unit (NICU). Compared to term infants, the preterm infant gut microbiome is less diverse, less stable, contains lower abundance of potentially probiotic bacteria, and a higher abundance of pathobiont bacteria. This altered gut microbiome has been associated with poorer health and an increased risk to several life-threatening diseases. Probiotics that contain *Bifidobacterium* species that can utilise HMOs have been used in many NICUs worldwide and overall meta-analyses suggests they can reduce disease risk, primarily relating to late onset sepsis (LOS) and necrotising enterocolitis (NEC). However, it would be desirable to create a probiotic formulation that is

more tailored to preterm infants to help improve growth and reduce disease risk. Such a probiotic might also have utility for improving health across the life course.

There is a need for novel compositions that improve the gut microbiome and reduces disease risk, particularly in respect of diseases exacerbated by probiotic imbalance.

Brief summary of the disclosure

The invention is based on the surprising finding that several bacterial species of the *Clostridium* genus utilise HMOs and therefore represent beneficial probiotics for improving the gut microbiome and/or gut health of a subject. The inventors have surprisingly discovered that *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum* utilise a number of HMOs when in co-culture, including at least one of: LNnT, LNT, 6SL, 2FL, DLSNT and LNFP_I. When grown on HMOs, HMO-utilising bacteria produce short chain fatty acids, convert aromatic amino acids into aromatic lactic acids (for example indolelactic acid (ILA)) and release other functionally important compounds that have significant beneficial immunomodulatory roles in the health of a subject. Interestingly, ILA has been shown to limit T cell activation and reduce a pro-inflammatory response by inducing galectin-1 in TH2 and TH17 cells during helper T cell polarisation. Accordingly, consumption of compositions that comprise one or more of these *Clostridium* species will therefore provide health benefits to a subject.

The inventors have also surprisingly shown that *Clostridium perfringens* is an abundant and prevalent species present in the gut in early life and is present at higher levels in healthy infants compared to infants diagnosed with necrotising enterocolitis (NEC) during the critical window of NEC risk (the first 10 to 40 days of life). Furthermore, the inventors have demonstrated that *Clostridium perfringens* is more abundant in the gut of healthy infants compared to infants before they were diagnosed with NEC, but not after recovery from NEC. This suggests that *Clostridium perfringens* plays an important role in protecting against NEC and suggests that increased levels of *Clostridium perfringens* in the gut would be beneficial and protective against disease.

The data provided herein indicates that a probiotic composition comprising one or more bacterial species of the *Clostridium* genus would improve the gut microbiome in a subject. Advantageously, all of *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum* have been shown to utilise at least one HMO, including LNnT, LNT, 6SL, 2FL, DSLNT and/or LNFP_I, thus sharing a common metabolic pathway and production of, for example, short chain fatty acids. The inventors have also shown that *Clostridium perfringens* is more abundant in the gut of healthy infants compared to infants before they

were diagnosed with NEC, which suggests that such a protective effect would be present for other diseases affecting, for example, the gastrointestinal tract. Notwithstanding the role of *Clostridium perfringens* in disease outcome for NEC, other therapeutic outcomes are dependent on probiotic levels and as such, it is also reasonable that one or more bacterial species of the *Clostridium* genus including *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum* would be therapeutically beneficial in preventing or treating any disorder that is exacerbated by probiotic imbalance, such as a gastrointestinal disorder, allergy, nappy rash, and colic, or a symptom thereof. By improving the gut microbiome and/or gut health in a subject, *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum* can advantageously reduce pathogen overgrowth in the gut, promote gut development, promote gut maturation, promote immunity, decrease gut inflammation, and/or promote enteral feeding tolerance in the subject.

Accordingly, in one aspect the invention provides a probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* further comprising at least one prebiotic; wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

Suitably, the composition may comprise at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

Suitably, the composition may comprise at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

Suitably, the composition may comprise at least 0.5×10^9 CFU *Clostridium* species per dose.

Suitably, the MMO may be a human milk oligosaccharide (HMO) a precursor and/or a derivative thereof.

Suitably, the HMO may be selected from the group consisting of: lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL) and 2-fucosyllactose (2FL); or a combination thereof; optionally wherein the HMO is LNnT and/or 6SL.

The invention also provides a postbiotic composition for improving the gut health of a subject, the composition comprising:

(a) at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or

(b) metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

Suitably, the postbiotic composition may comprise:

(a) at least two inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or

(b) metabolites from at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

Suitably, the postbiotic composition may comprise:

(a) at least three inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or

(b) metabolites from at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

Suitably, the probiotic composition or postbiotic composition may comprise:

a) a *Clostridium perfringens* species selected from the group consisting of: *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); and/or

b) the *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908); and/or

c) a *Clostridium tertium* species selected from the group consisting of: *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905) and *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911); and/or

d) a *Clostridium paraputrificum* species selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902) and *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910).

Suitably, the probiotic composition or postbiotic composition may be in the form of a liquid medium, gel medium, powder, dissolvable medium, chewable medium, or any other ingestible medium.

Suitably, the probiotic composition or postbiotic composition may be a milk product.

Suitably, the probiotic composition or postbiotic composition may be an infant formula, a starter infant formula, a follow-on or follow-up infant formula, a baby food, or an infant cereal composition.

Suitably, the probiotic composition or postbiotic composition may be a powder, a liquid or a solid.

The invention also provides a probiotic composition or postbiotic composition of the invention for use in improving the gut microbiome and/or the gut health of a subject.

Suitably, the subject may be an infant.

Suitably, the infant may be a neonate, a preterm infant, an infant small for gestational age, or an infant born by C-section.

Suitably, the infant may be hospitalised and/or is undergoing antibiotic treatment.

Suitably, the composition reduces pathogen overgrowth in the gut, promotes gut development, promotes gut maturation, promotes immunity, decreases gut inflammation, and/or promotes enteral feeding tolerance in the subject.

The invention also provides a probiotic composition or postbiotic composition of the invention for use in preventing or treating a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof, in a subject.

The invention also provides a probiotic composition for use in preventing or treating a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof, in a subject; wherein the probiotic composition comprises at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* and the subject consumes at least one prebiotic prior, after, or

during administration of the probiotic composition, wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

In other words, the invention also provides a probiotic composition for use in preventing or treating a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof, in a subject that consumes at least one prebiotic prior, after, or during administration of the probiotic composition; wherein the probiotic composition comprises at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* and wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

The invention also provides a method of treating or preventing a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof in a subject, comprising administering a therapeutically effective amount of the probiotic composition or the postbiotic composition of the invention to said subject.

The invention also provides a method of treating or preventing a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof in a subject, comprising administering a therapeutically effective amount of a probiotic composition prior, after, or during administration of a prebiotic composition, wherein the probiotic composition comprises at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* and wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

Suitably, the gastrointestinal disorder may affect the integrity of the intestinal lining.

Suitably, the gastrointestinal disorder may be selected from the group consisting of: intestinal inflammation, irritable bowel syndrome, colitis, necrotising enterocolitis, late onset sepsis, focal intestinal perforation, and inflammatory bowel disease.

Suitably, the gastrointestinal disorder may be necrotising enterocolitis.

Suitably, the subject may be an infant.

Suitably, the infant may be a neonate, a preterm infant, an infant small for gestational age, or an infant born by C-section.

Suitably, the infant may be hospitalised and/or is undergoing antibiotic treatment.

The invention also provides a probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected

from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*; wherein

a) the *Clostridium perfringens* species is selected from the group consisting of: *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); and/or

b) the *Clostridium baratti* species is *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908); and/or

c) the *Clostridium tertium* species is selected from the group consisting of: *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905) and *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911); and/or

d) the *Clostridium paraputrificum* species is selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902) and *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910).

The invention also provides the use of *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and/or *Clostridium paraputrificum* as a probiotic or postbiotic.

The invention also provides *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and/or *Clostridium paraputrificum,* or a *Clostridium* postbiotic thereof, for use in improving the gut microbiome and/or gut health in a subject.

The invention also provides a bacterial strain selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902), *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908), *Clostridium butyricum* strain JC31 (deposited under NCTC accession number 24022909),

Clostridium paraputrificum strain JC66 (deposited under NCTC accession number 24022910), *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); or a derivative thereof.

Various aspects of the invention are described in further detail below.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURES

Several strains of Clostridium species described in this application were deposited with the UK Health Security Agency (UKHSA) which is an International Depositary Authority located at Culture Collections, UK Health Security Agency, Porton Down, Salisbury, SP4 0JG, UK . The deposits were made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

Brief description of the Figures

Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

Figure 1 shows that several species of *Clostridium* and *Bifidobacterium* can use a range of HMOs. White squares containing "NA" in the DSLNT row indicate these co-cultures were not performed due to limited availability of DSLNT. Compared to Figure 1A, Figure 1B contains additional *Clostridium* species (*Clostridium paraputrificum*) that can use HMOs and is normalised to glucose.

Figure 2 shows *Clostridium perfringens* is the most abundant *Clostridium* species in early life and is higher in healthy infants compared to infants diagnosed with necrotising enterocolitis during the critical window of NEC risk. DOL: day of life.

Figure 3 shows *Bifidobacterium* is similar between healthy infants and infants diagnosed with necrotising enterocolitis during the critical window of NEC risk. DOL: day of life.

Figure 4 shows *Clostridium perfringens* is higher in healthy infants (NoNEC) compared to infants before they were diagnosed with necrotising enterocolitis (PreNEC), but not after recovery from NEC (PostNEC).

Figure 5 shows the postbiotic (i.e., conditioned medium) from the growth of *Clostridium perfringens* on HMOs was inhibitory to a range of pathobionts (*E. coli*, *E. faecalis*, *K. oxytoca*, *K. pneumoniae*, *E. cloacae* and *E. faecium*).

Figure 6 shows the postbiotic (i.e., conditioned medium) from the growth of *Clostridium tertium* on HMOs was inhibitory to a range of pathobionts (*E. coli*, *E. faecium*, *K. oxytoca*, *K. pneumoniae, E. cloacae, E. faecalis* and *S. haemolyticus*).

Figure 7 shows the postbiotic (i.e., conditioned medium) from the growth of *Clostridium perfringens* on HMOs promoted the growth of a range of beneficial *Bifidobacterium spp.* (*B. bifidum, B. breve* and *B. longum*). It also shows the postbiotic inhibited the growth of an adult-derived strain of *B. infantis*.

Figure 8 shows the postbiotic (i.e., conditioned medium) from the growth of *Clostridium tertium* on HMOs promoted the growth of a range of beneficial *Bifidobacterium spp.* (*B. bifidum, B. breve* and *B. longum*). It also shows the postbiotic inhibited the growth of an adult-derived strain of *B. infantis*.

Figure 9 shows that live *Clostridium perfringens* does not negatively impact the growth of *B. infantis* when the two species are cultured together.

Figure 10 shows that viabilities of human Caco-2 cells when incubated with increasing concentrations of postbiotics generated from *Clostridium* and *Bifidobacterium spp.* are comparable. Postbiotics derived from *Clostridium spp.* are shown to be no more toxic than those generated from *Bifidobacterium spp.* Letters at the top of the plot indicate outcomes of Tukey's statistical test performed at the genus level. Conditions with the same letters are not significantly different from one another.

Figure 11 shows the postbiotic from the growth of *Clostridium perfringens* on HMOs exerted protective effects on a human neonatal intestinal organoid monolayer. (A) Monolayer integrity, as measured by trans-epithelial resistance (TEER), was increased by the postbiotic post-treatment. The postbiotic also inhibited secretion of pro-inflammatory cytokines: IL8 apically (B) and basolaterally (C); CCL2 apically (D) and basolaterally (E); CCL7 apically (F) and basolaterally (G); TNFa apically (H) and basolaterally (I); CXCL5 basolaterally (J). Letters at the top of the plot indicate outcomes of Tukey's statistical test. Conditions with the same letters are not significantly different from one another.

Figure 12 shows the postbiotic from the growth of *B. infantis* on HMOs exerted similar, but weaker effects (compared to *Clostridium perfringens* (Figure 11)) on a human neonatal intestinal organoid monolayer. Panels appear in the same order as Figure 11. (A) Monolayer integrity, as measured by trans-epithelial resistance (TEER), was increased by the postbiotic post-treatment. The *B. infantis* postbiotic failed to inhibit apical secretion of pro-inflammatory IL8 (B), CCL7 (F), TNFa (H) and basolateral secretion of CXCL5 (J). But it did inhibit secretion of CCL2 apically (D) and basolaterally (E), as well as basolateral secretion of IL8

(C), CCL2 (E), CCL7 (G) and TNFa (I). Letters at the top of the plot indicate outcomes of Tukey's statistical test. Conditions with the same letters are not significantly different from one another.

Figure 13 A-G shows that when growing on HMOs, *Clostridium spp.* produce a greater quantity of the SCFAs propionate, butyrate, valerate and hexanoate than *Bifidobacterium spp.*, while *Bifidobacterium* spp. produced greater acetate. H and I further show that the global profile of *Clostridium*-produced SCFAs is distinct from that of *Bifidobacterium*-produced SCFAs, with *Clostridium* samples clustering separately from *Bifidobacterium* samples in both unsupervised ordination and dendrogram analyses. The ordination in H also emphasises that the SCFA profiles of *Clostridium spp.* grown on HMOs are more diverse than those of *Bifidobacterium spp.,* with a far wider spread of *Clostridium* samples based on the SCFA profiles.

Figure 14 shows PCA ordination and dendrogram plots of untargeted metabolomics data performed on glucose and HMO postbiotics, derived from both *Clostridium* and *Bifidobacterium*. Both plots show that samples largely cluster separately based on genera, indicating that *Clostridium* postbiotics contain a metabolite profile that is largely distinct from that of *Bifidobacterium*.

Figure 15 shows that *Clostridium* postbiotics contained detectable quantities of indole compounds and for five out of seven of these compounds, contained higher quantities than the *Bifidobacterium* postbiotics.

Figure 16 shows that various of the *Clostridium* postbiotics contained polyamines, diamines and their precursors. For many of these compounds (ornithine, citrulline, spermidine, agmatine, putrescine), *Clostridium* postbiotics contained equivalent or greater quantities than were measured in *Bifidobacterium* postbiotics.

Figure 17 shows three other metabolites of interest that in *Clostridium* postbiotics were either higher than (butyrate/isobutyrate and histamine) or equivalent to (phenyllactate) quantities detected in *Bifidobacterium* postbiotics.

Figure 18 shows that *Clostridium* postbiotics contained the neurotransmitters 3-hydroxybutyrate (A) and trimethylamine N-oxide (B). Both compounds were at higher quantities in some specific *Clostridium* postbiotics than were detected in the *Bifidobacterium* postbiotics.

The patent, scientific and technical literature referred to herein establish knowledge that was available to those skilled in the art at the time of filing. The entire disclosures of the issued patents, published and pending patent applications, and other publications that are cited

herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. In the case of any inconsistencies, the present disclosure will prevail.

Various aspects of the invention are described in further detail below.

Detailed Description

Probiotic compositions

The present invention relates to a probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*,*Clostridium tertium* and *Clostridium paraputrificum*.

The inventors have surprisingly identified that *Clostridium* species *Clostridium perfringens*, *Clostridium baratii* and *Clostridium tertium* and *Clostridium paraputrificum* are able to use a wide variety of HMOs as a carbon source. To the inventors' knowledge, this is the first time these species have been identified as probiotics for improving the gut microbiome and/or gut health, particularly in infants such as preterm infants.

The term "probiotic composition" as used herein refers to a composition comprising live bacteria that are beneficial to the overall health of an individual (referred to as a subject herein) that consumes the composition. Probiotic compositions are well known in the art. Probiotic compositions that are described herein impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome and/or gut health of the subject. Probiotic compositions may also be described as compositions that are utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease.

As would be clear to a person of skill in the art, a probiotic composition comprises sufficient quantities of the live bacteria to impart the required beneficial effect on the subject. The amount of bacteria in the probiotic composition may be referred to as a therapeutically effective amount. Appropriate amounts of live bacteria can readily be identified by a person skilled in the art. Further details and examples are provided elsewhere herein.

The probiotic potential of *Clostridium* species has been identified previously (see for example, Guo et al., 2020 (Journal of animal science and biotechnology); and Cartman, Future Microbiol. (2011), 6(9), 969-971). Although several *Clostridium* strains are considered to be pathogenic, *Clostridium* species account for 10-40% of the total bacteria in the human gut and are predominantly commensal in nature.

The probiotic compositions provided herein comprise live bacteria from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* or *Clostridium paraputrificum*. In one example, the probiotic compositions provided herein may comprise live bacteria from at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* or *Clostridium paraputrificum*. For example, the probiotic composition may comprise live *Clostridium perfringens* and *Clostridium baratii.* In another example, the probiotic composition may comprise live *Clostridium perfringens* and *Clostridium tertium*. In another example, the probiotic composition may comprise live *Clostridium perfringens* and *Clostridium paraputrificum.* In another example, the probiotic composition may comprise live *Clostridium baratii* and *Clostridium paraputrificum.* In another example, the probiotic composition may comprise live *Clostridium tertium* and *Clostridium paraputrificum.* In another example, the probiotic composition may comprise live *Clostridium baratii* and *Clostridium tertium*. In one example, the probiotic compositions provided herein may comprise live bacteria from at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* or *Clostridium paraputrificum*. In another example, the probiotic composition may comprise live *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

Clostridium (*Clostridiaceae*) is a genus of rod-shaped, anaerobic, chemoorganotrophic (ability to ferment), and spore-forming bacteria belonging to the *Bacillaceae* family of the phylum *Firmicutes*. Non-limiting examples of *Clostridium* species include *Clostridium perfringens, Clostridium baratii, Clostridium tertium*, *Clostridium paraputrificum, Clostridium butyricum, Clostridium botulinum, Clostridium sporogenes, Clostridium difficile, Clostridium tetani,* and *Clostridium ramosum*. Species of *Clostridium* are ubiquitous; naturally colonising the environmental medium (e.g. soil, water, and marine sediment) and the digestive tract of humans and animals. *Clostridium* species are a well-understood regulator of intestinal homeostasis. *Clostridium* species are Gram-variable, as they generally stain Gram-positive, however, as the culture ages, an increase in Gram-negative cells is often observable. Methods for performing a Gram stain are well known in the art.

Clostridium species primarily exist in three states: vegetative, spore-forming, and as an endospore. *Clostridium* species in either the vegetative or spore-forming states are considered "live" herein, as they are able to grow and reproduce. The term 'spore-forming' as used herein is intended to refer to *Clostridium* species at any stage of the sporulation cycle, except free endospore. The terms "spore(s)" and "endospore(s)" as used herein are interchangeable and refer to a dormant, tough, and non-reproductive free structure produced by the *Clostridium* species.

Clostridium perfringens is an obligate anaerobe, and is categorised into non-pathogenic and pathogenic strains, with the latter comprising seven types: A, B, C, D, E, F, and G. Non-pathogenic *Clostridium perfringens* strains comprise *Clostridium perfringens* strain SM101 and type A, and these are capable of producing secondary bile acids. Pathogenic *Clostridium perfringens* strains are frequently responsible for food poisoning and a large proportion gas gangrene cases.

The probiotic compositions described herein that comprise live *Clostridium perfringens* are beneficial to the overall health of an individual (referred to as a subject herein) that consumes the composition. The probiotic compositions described herein that comprise live *Clostridium perfringens* impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome of the subject. The probiotic compositions described herein that comprise live *Clostridium perfringens* may be utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease. In one example, the *Clostridium perfringens* is therefore a non-pathogenic strain, such as *Clostridium perfringens* strain SM101 and type A.

Several *Clostridium perfringens* strains have been characterised and the presence or absence of toxin expression and/or antimicrobial resistance (AMR) in these strains has been studied previously (see for example, Kiu et al., Dissemination and pathogenesis of toxigenic Clostridium perfringens strains linked to neonatal intensive care units and Necrotising Enterocolitis; 2021 bioRxiv preprint doi: https://doi.org/10.1101/2021.08.03.454877). A person of skill in the art can therefore readily identify non-pathogenic strains of *Clostridium perfringens.*

In some examples, the *Clostridium perfringens* provided herein is *pfoA*-negative. In other words, they do not express the pore-forming toxin perfringolysin O; PFO, which has been heavily associated with the pathogenesis of myonecrosis (gas gangrene).

In some examples, the *Clostridium perfringens* provided herein is a *cpb2*-negative. In other words, they do not express beta2-toxin, CPB2, which has been associated with virulence.

In some examples, the *Clostridium perfringens* provided herein does not comprise the plasmid pCW3 and/or pCP13, which have long been recognised as key mobile genetic elements for transfer of virulence genes, including toxins.

In some examples, the *Clostridium perfringens* provided herein is negative for one or more of the following toxin-associated genes: cpe, iap, ibp becA, becB and/or edpA.

The toxin genes pfoA, cpe, iap, ibp becA, becB and/or edpA are well known. For more details see for example Kiu et al., 2021, referenced above.

In some examples, the *Clostridium perfringens* provided herein is negative for one or more antimicrobial resistance genes selected from the group consisting of: cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. For example it may be negative for each of them (i.e. negative for all of the following): cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA In some examples, the *Clostridium perfringens* provided herein is negative for rpoB.

Clostridium baratii is an obligate anaerobe, and is rarely associated with human disease, however, when it is, it will usually occur after eating food contaminated with vegetative *Clostridium baratii* or its spores. *Clostridium baratii* can produce toxins which cause botulism, and is a rare cause of infant botulism.

The probiotic compositions described herein that comprise live *Clostridium baratii* are beneficial to the overall health of an individual (referred to as a subject herein) that consumes the composition. The probiotic compositions described herein that comprise live *Clostridium baratii* impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome of the subject. The probiotic compositions described herein that comprise live *Clostridium baratii* may be utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease. In one example, the *Clostridium baratii* is therefore a non-pathogenic strain.

Several *Clostridium baratii* strains have been characterised and the presence or absence of toxin expression and/or antimicrobial resistance (AMR) of these strains has been studied previously – see for example Silva-Andrade et al., Microorganisms. 2022 Feb; 10(2): 213. A person of skill in the art can therefore readily identify non-pathogenic strains of *Clostridium baratii*.

In some examples, the *Clostridium baratii* provided herein is negative for one or more toxin genes selected from the group consisting of: boNT, colA, NagH, NagI, NagJ, NagK, NagL, nanH, nanI, nanJ, tetX, and pfoA. For example it may be negative for each of them (i.e. negative for all of the following): boNT, colA, NagH, NagI, NagJ, NagK, NagL, nanH, nanI, nanJ, tetX, and pfoA. In some examples, the *Clostridium baratii* provided herein is negative for plc (alpha toxin phospholipase C).

NanH, nanI, and nanJ genes express sialidases. pfoA expresses theta-toxin/perfringolysin O. NagH, NagI, NagJ, nagK and NagL genes express mu-toxins, and colA expresses microbial collagenase. BoNT genes express botulinum toxins and tetX genes express tetanus toxin. For more information see for example Silva-Andrade et al., Microorganisms. 2022 Feb; 10(2): 213.

In some examples, the *Clostridium baratii* provided herein is negative for one or more antimicrobial resistance genes selected from the group consisting of: cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. For example it may be negative for each of them (i.e. negative for all of the following): cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. In some examples, the *Clostridium baratii* provided herein is negative for rpoB.

The antimicrobial genes cfrC, ET-Tu, gyrA, gryB, rpoC, TetA and rpoB are well characterised. For more details see for example Silva-Andrade et al., Microorganisms. 2022 Feb; 10(2): 213.

Clostridium tertium is both anaerobic and aerotolerant. Although the normal habitat of *Clostridium tertium* is the soil, it is commonly found as a commensal colonising the digestive tract of humans and animals. Although rarely pathogenic, it is thought to be associated with enterocolitis in preterm neonates.

The probiotic compositions described herein that comprise live *Clostridium tertium* are beneficial to the overall health of an individual (referred to as a subject herein) that consumes the composition. The probiotic compositions described herein that comprise live *Clostridium tertium* impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome of the subject. The probiotic compositions described herein that comprise live *Clostridium tertium* may be utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease. In one example, the *Clostridium tertium* is therefore a non-pathogenic strain.

Several *Clostridium tertium* strains have been characterised and the presence or absence of toxin expression and/or antimicrobial resistance (AMR) of these strains has been studied previously. A person of skill in the art can therefore readily identify non-pathogenic strains of *Clostridium tertium*.

In some examples, the *Clostridium tertium* provided herein is negative for one or more antimicrobial resistance genes selected from the group consisting of: cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. For example it may be negative for each of them (i.e. negative for all of the following): cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. In some examples, the *Clostridium tertium* provided herein is negative for rpoB.

Clostridium paraputrificum is a anaerobic, motile, gram-positive bacterium that is considered part of the normal intestinal microbiome. It is rarely associated with human disease but has been reported in bacteremia.

The probiotic compositions described herein that comprise live *Clostridium paraputrificum* are beneficial to the overall health of an individual (referred to as a subject herein) that consumes the composition. The probiotic compositions described herein that comprise live *Clostridium*

paraputrificum impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome of the subject. The probiotic compositions described herein that comprise live *Clostridium paraputrificum* may be utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease. In one example, the *Clostridium paraputrificum* is therefore a non-pathogenic strain.

Several *Clostridium paraputrificum* strains have been characterised and the presence or absence of toxin expression and/or antimicrobial resistance (AMR) of these strains has been studied previously. A person of skill in the art can therefore readily identify non-pathogenic strains of *Clostridium paraputrificum*.

In some examples, the *Clostridium paraputrificum* provided herein is negative for one or more antimicrobial resistance genes selected from the group consisting of: cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. For example it may be negative for each of them (i.e. negative for all of the following): cfrC, ET-Tu, gyrA, gryB, rpoC, and TetA. In some examples, the *Clostridium paraputrificum* provided herein is negative for rpoB.

Clostridium paraputrificum strain JC53 (24022902), *Clostridium perfringens* strain AM1 (24022903), *Clostridium perfringens* strain JC26 (24022904), *Clostridium tertium* strain JC25 (24022905), *Clostridium perfringens* strain AM248 (24022906), *Clostridium perfringens* strain JC36 (24022907), *Clostridium baratti* strain AM37 (24022908), *Clostridium butyricum* strain JC31 (24022909), *Clostridium paraputrificum* strain JC66 (24022910), *Clostridium tertium* strain TB43 (24022911), *Clostridium perfringens* strain JC33 (24022912), *Clostridium perfringens* strain AM226 (24022913), and *Clostridium perfringens* strain JC29 (24022914) have been found to be particularly important in the context of the present invention.

A *Clostridium paraputrificum* strain deposited under NCTC accession number 24022902 may be referred to herein as “JC53” or “strain JC53”. *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022903 may be referred to herein as “AM1” or “strain AM1”. *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022904 may be referred to herein as “JC26” or “strain JC26”. *Clostridium perfringens* strain JC26 (deposited

under NCTC accession number 24022904) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium tertium* strain deposited under NCTC accession number 24022905 may be referred to herein as "JC25" or "strain JC25". *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022906 may be referred to herein as "AM248" or "strain AM248". *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022907 may be referred to herein as "JC36" or "strain JC36". *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium baratti* strain deposited under NCTC accession number 24022908 may be referred to herein as "AM37" or "strain AM37". *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium butyricum* strain deposited under NCTC accession number 24022909 may be referred to herein as "JC31" or "strain JC31". *Clostridium butyricum* strain JC31 (deposited under NCTC accession number 24022909) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium paraputrificum* strain deposited under NCTC accession number 24022910 may be referred to herein as "JC66" or "strain JC66". *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium tertium* strain deposited under NCTC accession number 24022911 may be referred to herein as "TB43" or "strain TB43". *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022912 may be referred to herein as "JC33" or "strain JC33". *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022913 may be referred to herein as "AM226" or "strain AM226". *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

A *Clostridium perfringens* strain deposited under NCTC accession number 24022914 may be referred to herein as "JC29" or "strain JC29". *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914) was deposited at Culture Collections, UK Health Security Agency (Porton Down, Salisbury, SP4 0JG, UK). This strain was deposited under The Budapest Treaty of 1977 on 29 February 2024.

Accordingly, in some examples the *Clostridium paraputrificum* species may be selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (24022902) and *Clostridium paraputrificum* strain JC66 (24022910).

Accordingly, in some examples the *Clostridium perfringens* species may be selected from the group consisting of: *Clostridium perfringens* strain AM1 (24022903), *Clostridium perfringens* strain JC26 (24022904), *Clostridium perfringens* strain AM248 (24022906), *Clostridium perfringens* strain JC36 (24022907), *Clostridium perfringens* strain JC33 (24022912), *Clostridium perfringens* strain AM226 (24022913), and *Clostridium perfringens* strain JC29 (24022914).

Accordingly, in some examples the *Clostridium tertium* species may be selected from the group consisting of: *Clostridium tertium* strain JC25 (24022905) and *Clostridium tertium* strain TB43 (24022911).

Accordingly, in some examples the *Clostridium baratti* species may be *Clostridium baratti* strain AM37 (24022908).

Accordingly, in some examples the *Clostridium butyricum* species may be *Clostridium butyricum* strain JC31 (24022909).

Probiotic bacteria may be combined with a prebiotic. They can be combined into a single composition that comprises both the probiotic bacteria and at least one prebiotic such that they are consumed simultaneously by the subject (in other words, a probiotic composition provided herein may further comprise a prebiotic as well). Alternatively, they may be in separate (distinct) compositions, whereby the probiotic composition of the invention and a prebiotic composition are consumed separately (simultaneously or consecutively) by the subject.

Accordingly, in the present invention, at least one prebiotic is present within the probiotic composition. In another example, at least one prebiotic is provided as a separate prebiotic composition (separate from the probiotic composition) and is administered to the subject either prior, after, or during administration of the probiotic composition. Examples of such prebiotic compositions are described elsewhere herein.

A probiotic composition is therefore provided, wherein the probiotic bacteria are combined with at least one prebiotic within the same composition. Such compositions may also be referred to herein as a "synbiotic" composition.

A prebiotic composition is also provided, which lacks the probiotic bacteria described herein (i.e. lacks *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*), wherein the prebiotic composition is for separate (simultaneous or consecutive) administration to the same subject as the probiotic composition of the invention. Details of the prebiotic composition are provided elsewhere herein.

The term "prebiotic" as used herein refers to a component that promotes the growth of probiotic bacteria. In certain instances, prebiotic may refer to food ingredients or bacterially produced substances that are not readily digestible by endogenous host enzymes (in other words, the prebiotic is not digestible by the subject that consumes the composition of the invention, but rather by the probiotic bacteria that may colonise its gut). Prebiotics may originate from several sources, such as artichokes, asparagus, bananas, berries, tomatoes, garlic, onions, legumes, green vegetables, wholegrain cereals, and milk, such as breast milk or cows' milk. Prebiotics may for example be MMOs (mammalian milk oligosaccharides) such as HMOs (human milk oligosaccharides). In certain instances, prebiotics may be synthetic in origin or manufactured artificially.

As chemoorganotrophic bacteria, *Clostridium* species are able to ferment several nutrients including, but not limited to, protein, carbohydrate, organic acid, and non-digestible substrates

(such as oligosaccharides), to generate short-chain fatty acids (SCFAs) such as propionic acid, butyric acid, acetic acid, and solvents such as acetone and butanol.

The *Clostridium* species *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum* are able to use a wide variety of prebiotics, in particular MMOs such as HMOs. This makes them to useful probiotics that can improve the gut microbiome and/or gut health, particularly in infants that readily consume milk (e.g. in the form of infant formula, breast milk etc) that readily has MMOs, such as HMOs therein. This leads to the production of short chain fatty acids, conversion of aromatic amino acids into aromatic lactic acids, for example indolelactic acid (ILA) and the release of other functionally important compounds that have significant immunomodulatory roles in the health of a subject.

The probiotic composition of the invention comprises at least one prebiotic. In one example it comprises at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen etc prebiotics.

In the present invention, the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

MMO, as used herein, refers to indigestible glycans, sometimes referred to as “dietary fiber”, or carbohydrate polymers found in mammalian milk which are not metabolized by any combination of mammalian digestive enzymes. Mammalian milk contains a significant quantity of MMO that is not usable directly as an energy source for the milk-fed mammal but may be usable by many of the microorganisms in the gut of that mammal. MMOs can be found as free oligosaccharides (3 sugar units or longer, e.g., 3-20 sugar residues) or they may be conjugated to proteins or lipids. Oligosaccharides having the chemical structure of the indigestible oligosaccharides found in any mammalian milk are called “MMO” or “mammalian milk oligosaccharides” herein, whether or not they are actually sourced from mammalian milk. The MMO can include one or more of fucosyllactose, lacto-N-fucopentose, lactodifucotetrose, sialyllactose, disialyllacto-N-tetraose, 2′-fucosyllactose, 3′-sialyllactoseamin, 3′-fucosyllactose, 3′-sialyl-3-fucosyllactose, 3′-sialyllactose, 6′-sialyllactosamine, 6′-sialyllactose, difucosyllactose, lacto-N-fucosylpentose I, lacto-N-fucosylpentose II, lacto-N-fucosylpentose III, lacto-N-fucosylpentose V, sialyllacto-N-tetraose, or derivatives thereof. See, e.g., U.S. Pat. Nos. 8,197,872, 8,425,930, and 9,200,091, the disclosures of which are incorporated herein by reference in their entirety.

Several MMOs are well known in the art, including that from human milk (HMO), bovine milk (BMO), ovine milk (OMO), equine milk (EMO) or caprine milk (CMO). The oligosaccharides can be obtained from a process that involves cheese or yogurt production and can be from

whey sources such as, but not limited to, the whey permeate, or a processed whey permeate, where the processing steps may include, but are not limited to, removal of lactose, removal of minerals, removal of peptides, and removal of monosaccharides, but which in any case, results in the concentration of the MMO to levels that are greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, or greater than 80% of the total dry matter of the product.

In the present invention, the probiotic composition of the invention comprises at least one MMO. In other example, a prebiotic composition comprising at least one MMO is administered to the subject as a separate (distinct) composition to the probiotic composition of the invention, whereby the probiotic composition of the invention and a prebiotic composition are consumed separately (simultaneously or consecutively) by the subject. In this context, the prebiotic composition comprising at least one MMO may be a milk selected from the group consisting of human milk (HMO), bovine milk (BMO), ovine milk (OMO), equine milk (EMO) or caprine milk (CMO).

In one example, the prebiotic is a human milk oligosaccharide (HMO), a precursor and/or a derivative thereof.

HMOs are a complex mixture of more than 200 non-digestible and non-nutritional carbohydrate polymers, which are not metabolised by any combination of human digestive enzymes in the digestive tract. These HMOs occur naturally in human breast milk only. As they are non-digestible, the subject is unable to utilise them as an energy source. However, HMOs are a bonafide energy source for several probiotic members of the gut microbiota in the subject. After lactose and lipids, HMOs are the third most abundant component of breast milk. Human breast milk contains three major HMO types: fucosylated HMOs, sialylated HMOs, and nonfucosylated neutral HMOs, which typically make up 35-50%, 12-14%, and 42-55% of the total HMO content of milk, respectively. HMOs are made of five basic monosaccharides: D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and *N-Acetylneuraminic acid (*sialic acid). The majority of HMOs are based on a lactose molecule at the reducing end, and branching can be linear or branched through beta (1-3) or beta (1-6) bonds. Non-limiting examples of exemplary HMOs include lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL), and 2-fucosyllactose (2FL). These HMOs are well known and are well characterised in the art. HMOs are virtually absent in non-human milk and in unsupplemented infant formula.

The HMO used for promoting colonisation and/or growth of probiotic bacteria present in the probiotic compositions described herein can include fucosylated HMOs, precursors, or

derivatives thereof. Accordingly, the probiotic compositions provided herein may comprise, or may be administered in combination with, a fucosylated HMO, precursor or derivative thereof (prebiotic). Non-limiting examples of fucosylated HMOs include 2FL, LNFPI, and LDFH I which can be purified from human milk or produced directly from chemical synthesis. 2FL is the most abundant HMO, constituting nearly 30% of the total HMOs in human breast milk.

The HMO used for promoting colonisation and/or growth of the probiotic bacteria present in the probiotic compositions described herein can include sialylated HMOs, precursors or derivatives thereof. Accordingly, the probiotic compositions provided herein may comprise, or may be administered in combination with, a sialylated HMO, precursor or derivative thereof (prebiotic). Non-limiting examples of sialylated HMO derivatives include DSLNT, 3SL, 6SL, LSTa, LSTb, LSTc.

The HMO used for promoting colonisation and/or growth of the probiotic bacteria present in the probiotic compositions described herein can include nonfucosylated neutral HMOs, precursors or derivatives thereof. Accordingly, the probiotic compositions provided herein may comprise, or may be administered in combination with, a nonfucosylated neutral HMO, precursor or derivative thereof (prebiotic). Non-limiting examples of nonfucosylated neutral HMOs include LNT, LNnT and LNnH.

In one example, the HMO that is present in the probiotic composition of the invention is selected from the group consisting of: lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL) and 2-fucosyllactose (2FL); or a combination thereof.

In one example, the HMO that is present in the probiotic composition of the invention is LNnT and/or 6SL.

In one example, the probiotic composition of the invention comprises at least one HMO. In other example, a prebiotic composition comprising at least one HMO is administered to the subject as a separate (distinct) composition to the probiotic composition of the invention, whereby the probiotic composition of the invention and a prebiotic composition are consumed separately (simultaneously or consecutively) by the subject.

The abundance of specific HMOs in human milk is variable, and may change based on the genetics, diet, physiological state of the mother, pathological state of the mother, and/or psychological state of the mother. The HMO composition received by the subject at a certain time point may reflect that of a healthy mother at the corresponding time point.

In one example, a plurality of HMOs are present within the probiotic composition of the invention. For example, the probiotic composition may comprise at least two, at least three, at least four, at least five, at least six etc HMOs.

The HMOs present in the probiotic composition of the invention may be a mixture of lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL), and/or 2-fucosyllactose (2FL), which are all naturally found in human milk.

In one example, the probiotic composition of the invention comprises human milk.

In other example, human milk is administered to the subject as a separate (distinct) composition to the probiotic composition of the invention, whereby the probiotic composition of the invention and a prebiotic composition are consumed separately (simultaneously or consecutively) by the subject.

In a further example, the probiotic composition of the invention comprises at least one HMO precursor. The term "HMO precursor" as used herein refers to mono-, di- and trisaccharides that are the parts of HMOs, that is glucose, galactose, N-acetyl-glucosamine, fucose, sialic acid, lactose, lacto-N-biose (Gaipi-3GlcNAc), N-acetyl-lactosamine (Gaipi-4GlcNAc) and lacto-N- triose (GlcNAcpi-3Gaipi-4Glc). Preferred HMO precursors are selected from the group consisting of fucose, sialic acid, lacto-N-biose, N-acetyl-lactosamine and lacto-N-triose.

In one example, the probiotic composition of the invention comprises at least one HMO precursor. In other example, a prebiotic composition comprising at least one HMO precursor is administered to the subject as a separate (distinct) composition to the probiotic composition of the invention, whereby the probiotic composition of the invention and a prebiotic composition are consumed separately (simultaneously or consecutively) by the subject.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and at least one prebiotic. For example, *Clostridium perfringens* and LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and LNnT, 6SL, 2FL, DSLNT, and/or LNT.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and LNnT, 6SL, 2FL, and/or DSLNT.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and LNnT, 6SL and/or DSLNT.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and LNnT and/or 6SL.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and LNnT.

In one example, the probiotic composition of the invention comprises *Clostridium perfringens* and 6SL.

In one example, the probiotic composition of the invention comprises *Clostridium baratii* and at least one prebiotic. For example, *Clostridium baratii* and LNnT, and/or LNT.

In one example, the probiotic composition of the invention comprises *Clostridium baratii* and LNnT.

In one example, the probiotic composition of the invention comprises *Clostridium baratii* and LNT.

In one example, the probiotic composition of the invention comprises *Clostridium tertium* and at least one prebiotic. For example, *Clostridium tertium* and LNnT, and/or LNT.

In one example, the probiotic composition of the invention comprises *Clostridium tertium* and LNnT.

In one example, the probiotic composition of the invention comprises *Clostridium tertium* and LNT.

In one example, the probiotic composition of the invention comprises *Clostridium paraputrificum* and at least one prebiotic. For example, *Clostridium paraputrificum* and LNnT, and/or LNT.

In one example, the probiotic composition of the invention comprises *Clostridium paraputrificum* and LNnT.

In one example, the probiotic composition of the invention comprises *Clostridium paraputrificum* and LNT.

The probiotic composition may comprise a two or more *Clostridium* species selected from the group consisting of *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum.* The probiotic composition may comprise two or more *Clostridium* species and at least one prebiotic.

For example, it may comprise *Clostridium perfringens* and *Clostridium baratii* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT.

For example, it may comprise *Clostridium perfringens* and *Clostridium baratii* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium perfringens* and *Clostridium tertium* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium perfringens* and *Clostridium tertium* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium tertium* and *Clostridium baratii* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium tertium* and *Clostridium baratii* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium perfringens* and *Clostridium paraputrificum* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium perfringens* and *Clostridium paraputrificum* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium tertium* and *Clostridium paraputrificum* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium tertium* and *Clostridium paraputrificum* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium baratii* and *Clostridium paraputrificum* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium baratii* and *Clostridium paraputrificum* and at least one prebiotic selected from LNnT, and/or LNT.

For example, it may comprise *Clostridium perfringens, Clostridium tertium, Clostridium baratii* and *Clostridium paraputrificum* and at least one prebiotic selected from the group consisting of: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT. For example, it may comprise *Clostridium perfringens, Clostridium tertium, Clostridium baratii* and *Clostridium paraputrificum* and at least one prebiotic selected from LNnT, and/or LNT.

In some examples, the probiotic composition may comprise additional bacteria, such as *Bifidobacterium*. Accordingly, the probiotic composition may comprise *Bifidobacterium* and one or more Clostridium species selected from the group consisting of *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum.*

Prebiotic composition

A prebiotic composition is also provided, which comprises at least one prebiotic and lacks the probiotic bacteria described herein (i.e. lacks *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*), wherein the prebiotic composition is for separate (simultaneous or consecutive) administration to the same subject as the probiotic composition of the invention.

Examples of prebiotics that may be included in the prebiotic composition are provided above and apply equally to this aspect.

The prebiotic composition comprises at least one prebiotic. In one example it comprises at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen etc prebiotics.

In one example, in the prebiotic composition, the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof. In one example, the prebiotic is a human milk oligosaccharide (HMO), a precursor and/or a derivative thereof. These terms are defined elsewhere herein and apply equally to this aspect.

The HMO may be a fucosylated HMO, precursor, or derivative thereof. In another example, the HMO may be a sialylated HMO, precursor, or derivative thereof. In another example, the HMO may be a nonfucosylated neutral HMO, precursor, or derivative thereof. Non-limiting examples of these HMOs are provided elsewhere herein.

In one example, the HMO that is present in the prebiotic composition is selected from the group consisting of: lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL) and 2-fucosyllactose (2FL); or a combination thereof.

In one example, the HMO that is present in the prebiotic composition is LNnT and/or 6SL.

In one example, a plurality of HMOs are present within the prebiotic composition of the invention. For example, the prebiotic composition may comprise at least two, at least three, at least four, at least five, at least six etc HMOs.

The HMOs present in the prebiotic composition may be a mixture of lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL), and/or 2-fucosyllactose (2FL), which are all naturally found in human milk.

In one example, the prebiotic composition comprises a milk selected from the group consisting of: human milk (HMO), bovine milk (BMO), ovine milk (OMO), equine milk (EMO) or caprine milk (CMO).

In one example, the prebiotic composition comprises human milk.

In a further example, the prebiotic composition comprises at least one HMO precursor. Non-limiting examples of HMO precursors are provided elsewhere herein.

Postbiotic composition

The invention also provides a postbiotic composition for improving the gut health of a subject, the composition comprising:

(a) at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or

(b) metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

In some examples, the postbiotic composition may further comprise:

(a) at least one inanimate additional bacterial species, such as *Bifidobacterium*; and/or

(b) metabolites of at least one additional bacterial species, such as *Bifidobacterium*.

Accordingly, the postbiotic composition for improving the gut health of a subject may comprise:

(a) at least one inanimate *Bifidobacterium* species and at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*; and/or

(b) metabolites from at least one *Bifidobacterium* species and metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

The term "postbiotic composition" as used herein is a general term to refer to a preparation of inanimate probiotic bacteria and/or their component parts (e.g., cellular debris) and/or their metabolites, wherein the composition is beneficial to the overall health of an individual (referred to as a subject herein) that consumes it. Postbiotic compositions are well known in the art. Postbiotic compositions that are described herein impart their beneficial effects in the gut of the subject, and therefore improve the gut microbiome of the subject. Postbiotic

compositions may also be described as compositions that are utilised as a dietary supplement to alter the digestive tract microbiota of a subject in a beneficial manner without causing disease.

As would be clear to a person of skill in the art, a postbiotic composition comprises sufficient quantities of the inanimate probiotic bacteria (and/or their component parts (e.g., cellular debris) and/or their metabolites) to impart the required beneficial effect on the subject. Preparations of postbiotics typically contain the inanimate probiotic bacteria and can also retain microbe-produced substances, such as metabolites, proteins, or peptides, which may contribute to the overall health effect conferred by a postbiotic, but such microbe-produced substances are not essential to a postbiotic. The amount of inanimate probiotic bacteria (and/or their component parts (e.g., cellular debris) and/or their metabolites) in the postbiotic composition may be referred to as a therapeutically effective amount. Appropriate amounts can readily be identified by a person skilled in the art. Further details and examples are provided elsewhere herein.

The term “inanimate” as used herein refers to a bacterium that is no longer alive, or, in other words, dead or inactivated, and may comprise non-viable whole cells, cellular fragments, proteins, or peptides. Components derived from inanimate microorganisms may comprise by-products such as metabolites. Appropriate metabolites are well known in the art and include SCFAs (such as butyrate, acetate, and/or propionate), secondary bile acid, and tryptophan metabolites (such as ILA).

Postbiotic compositions are provided herein which comprise at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium and/or Clostridium paraputrificum* or at least one *Clostridium perfringens* metabolite*, Clostridium baratii* metabolite*, Clostridium tertium* metabolite *and/or Clostridium paraputrificum* metabolite.

In one example, the postbiotic composition comprises at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium and/or Clostridium paraputrificum*. The composition is beneficial to the overall health of an individual (referred to as a subject herein) that consumes it. The postbiotic composition comprising at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium and/or Clostridium paraputrificum* imparts its beneficial effects in the gut of the subject, and therefore improves the gut health of the subject.

The postbiotic composition may comprise at least two inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium*

and/or Clostridium paraputrificum. For example, the postbiotic composition may comprise inanimate *Clostridium perfringens* and inanimate *Clostridium baratii.* In another example, the postbiotic composition may comprise inanimate *Clostridium perfringens* and inanimate *Clostridium tertium*. In another example, the postbiotic composition may comprise inanimate *Clostridium perfringens* and inanimate *Clostridium paraputrificum*. In another example, the postbiotic composition may comprise inanimate *Clostridium paraputrificum* and inanimate *Clostridium tertium*. In another example, the postbiotic composition may comprise inanimate *Clostridium paraputrificum* and inanimate *Clostridium baratii.* In another example, the postbiotic composition may comprise inanimate *Clostridium baratii* and inanimate *Clostridium tertium*. In another example, the postbiotic composition may comprise at least three inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium and/or Clostridium paraputrificum*. In another example, the postbiotic composition may comprise inanimate *Clostridium perfringens,* inanimate *Clostridium baratii*, inanimate *Clostridium tertium* and inanimate *Clostridium paraputrificum*.

The *Clostridium* can for example be made inanimate by killing at high temperature and pressure. It can for example also be removed by sterile filtration. Such methods are well known in the art.

In another example, the postbiotic composition comprises metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*. The composition is beneficial to the overall health of an individual (referred to as a subject herein) that consumes it. The postbiotic composition comprising metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum* imparts its beneficial effects in the gut of the subject, and therefore improves the gut health of the subject.

The postbiotic composition may comprise metabolites from at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum*. For example, the postbiotic composition may comprise metabolites from *Clostridium perfringens* and *Clostridium baratii.* In another example, the postbiotic composition may comprise metabolites from *Clostridium perfringens* and *Clostridium tertium*. In another example, the postbiotic composition may comprise metabolites from *Clostridium perfringens* and *Clostridium paraputrificum*. In another example, the postbiotic composition may comprise metabolites from *Clostridium paraputrificum* and *Clostridium tertium*. In another example, the postbiotic composition may comprise metabolites from *Clostridium paraputrificum* and *Clostridium baratii*. In another example, the postbiotic

composition may comprise metabolites from *Clostridium baratii* and *Clostridium tertium*. In another example, the postbiotic composition may comprise metabolites from at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum*. In another example, the postbiotic composition may comprise metabolites from *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

The terms "*Clostridium perfringens* metabolite", *"Clostridium baratii* metabolite", *"Clostridium tertium* metabolite", and *"Clostridium paraputrificum* metabolite" as used herein refer to metabolites originating from the metabolic activity of these *Clostridium* species, respectively, in particular when grown on one or more HMOs as a carbon source.

A *Clostridium perfringens* metabolite, *Clostridium baratii* metabolite, *Clostridium tertium* metabolite and/or *Clostridium paraputrificum* metabolite can for example be a short chain fatty acid (such as butyrate, acetate, and/or propionate), secondary bile acid, and/or tryptophan metabolites (such as ILA).

As would be clear to a person of skill in the art, a postbiotic composition may comprise metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium,* and/or *Clostridium paraputrificum* due to the postbiotic composition being an in vitro cell culture product. In other words, the postbiotic composition may be generated by in vitro cell culture of the at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum.* In vitro cell culture of the at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum* may be for a sufficient time period (and under appropriate conditions) to generate metabolites from the one or more *Clostridium* species. Typically, the in vitro cell culture of the at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum* may be performed by culturing the at least one *Clostridium* species with a prebiotic for a sufficient time period (and under appropriate conditions) to generate the metabolites. For example, the in vitro cell culture of the at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or *Clostridium paraputrificum* may be performed by culturing the at least one *Clostridium* species with a MMO for a sufficient time period (and under appropriate conditions) to generate the metabolites.

In one example, the in vitro cell culture of the at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and/or

Clostridium paraputrificum may be performed by culturing the at least one *Clostridium* species with a HMO for a sufficient time period (and under appropriate conditions) to generate the metabolites.

The postbiotic composition may then be generated by removing the *Clostridium* species from the in vitro cell culture after the metabolites have been generated. Any appropriate method may be used to remove the *Clostridium* species, for example filtration, centrifugation etc.

A non-limiting example of how to generate an appropriate postbiotic is provided in the examples section below.

A postbiotic composition may therefore be generated by culturing the appropriate *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum* anaerobically in the presence of a suitable MMO under appropriate culture conditions.

For example, *Clostridium perfringens* may be cultured in the presence of a HMO selected from: LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, DSLNT, and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, and/or DSLNT under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL and/or DSLNT under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of a HMO selected from*:* LNnT and/or 6SL under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of LNnT under appropriate culture conditions. For example, *Clostridium perfringens* may be cultured in the presence of 6SL under appropriate culture conditions.

For example, *Clostridium baratii* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions. For example, *Clostridium baratii* may be cultured in the presence of LNnT. For example, *Clostridium baratii* may be cultured in the presence of LNT under appropriate culture conditions.

For example, *Clostridium tertium* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions. For example, *Clostridium tertium* may be cultured in the presence of LNnT. For example, *Clostridium tertium* may be cultured in the presence of LNT under appropriate culture conditions.

For example, *Clostridium paraputrificum* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions. For example, *Clostridium*

paraputrificum may be cultured in the presence of LNnT. For example, *Clostridium paraputrificum* may be cultured in the presence of LNT under appropriate culture conditions.

For example, *Clostridium perfringens* and *Clostridium baratii* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens* and *Clostridium baratii* may be cultured in the presence of a HMO selected from: LNnT, and/or LNT under appropriate culture conditions.

For example, *Clostridium perfringens* and *Clostridium tertium* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens* and *Clostridium tertium* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions.

For example, *Clostridium perfringens* and *Clostridium paraputrificum* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens* and *Clostridium paraputrificum* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions.

For example, *Clostridium perfringens, Clostridium tertium, Clostridium baratii* and *Clostridium paraputrificum* may be cultured in the presence of a HMO selected from*:* LNnT, 6SL, 2FL, DSLNT, LNFP-I and/or LNT under appropriate culture conditions. For example, *Clostridium perfringens, Clostridium tertium, Clostridium baratii* and *Clostridium paraputrificum* may be cultured in the presence of a HMO selected from*:* LNnT, and/or LNT under appropriate culture conditions.

Appropriate culture conditions will be readily identifiable to a person of skill in the art (e.g. anaerobically at between 35 – 39°C, for a period of at least 2 days, typically at least 5 or 6 days), optionally whilst shaking (e.g. at 130 rpm).

Appropriate culture conditions include anaerobic culture at around 37°C, for a period of at least 2 days, typically at least 5 or 6 days), optionally whilst shaking (e.g. at 130 rpm).

The postbiotic composition may then be generated by removing the *Clostridium* species from the cell culture. Any appropriate method may be used to remove the *Clostridium* species, for example filtration, centrifugation etc. Centrifugation may be for at least 5000g for at least 5 minutes at about 4°C. Fitration may be using a 0.22 µm filter. Centrifugation for at least 5000g for at least 5 minutes at about 4°C may be combined with fitration using a 0.22 µm filter.

The postbiotic composition may alternatively be generated by heat treating the *Clostridium* species in the cell culture to ensure that they are inanimate.

The postbiotic composition may include the entire cell culture, with the exception of live *Clostridium* species.

Formulation of the composition

The compositions provided herein may be administered to the subject in any suitable format and by any appropriate means. Typically, the compositions described herein are for oral use. Accordingly, the compositions described herein are typically comestible (also referred to as edible herein). Suitably, the composition may be in the form of a liquid medium, gel medium, powder, dissolvable medium, chewable medium, or any other ingestible medium. This includes, without limitation, tablets, capsules (including a gelatin capsules), pills, caplets, yogurts, freeze dried products, granules, syrups, drinks, food and the like. Suitable ways of preparing such compositions are well known in the art.

In one example the composition is in a powder, liquid or solid form.

Compositions described herein may also contain conventional food supplement fillers and extenders such as, for example, a flour, a binder, a neutraceutical compound or formulation, an amino acid, a vitamin, or a mineral.

The bacteria described herein may be dried with a food product as described in WO98/10666, which is incorporated herein by reference in its entirety. Accordingly, the *Clostridium* species may be dried at the same time with juices, milk-based products or vegetable milks, for example, yielding a dried product already comprising the *Clostridium* species. This product may later be reconstituted with an aqueous liquid such as milk or water.

The composition may be a milk product. A milk product is any comestible composition that comprises animal milk (obtained from a mammal) or a milk substitute (such as plant-based milk products). The milk product may be in liquid, solid or powder form.

A milk product may comprise animal milk or a milk substitute. Examples of animal milk include, but are not limited to, bovine, goat, sheep, camel, donkey, and reindeer milk. Human milk is also encompassed by this term. A non-human or non-animal milk product may also be referred to as a “milk substitute” herein. Milk substitutes therefore include plant milk, such as almond, soy, oat, coconut, pea, hemp, spelt, rice, pistachio, walnut, macadamia, flax, banana, cashew, hazelnut, quinoa, and sesame milk. Milk substitutes also include modified milk, such as infant formula.

A milk product includes, but is not limited to, cheese, butter, cream, yoghurt, ghee, condensed milk, ice cream, buttermilk, milkshake, and whey etc. A milk product also includes milk-based drinks or powders; and drinks or powders based on milk substitutes.

The milk product may comprise human donor breast milk.

The milk product may also be a milk product that has been generated in vitro. For example, it may be milk that has been produced by mammary organoids.

The milk product may be a modified milk product. In other words, instead of comprising naturally occurring milk, it may comprise modified milk that has been adapted for the subject of interest. An example of a modified milk product is infant formula, which comprises cow's milk that has been treated to make it more suitable for the subject of interest (e.g. human infants).

In one example, the composition is an infant milk product that is selected from the group consisting of: infant formula, a starter infant formula, a follow-on or follow-up infant formula, a baby food, or an infant cereal composition.

As used herein, the terms "formula milk", "formula", "baby formula", "growing-up milk", or "infant formula" are interchangeable and are intended to represent the substitute for human or animal breast milk which is given to infant humans or animals (0-36 months of age). The infant formula may be powdered, concentrated liquid formula, or ready-to-use formula.

A milk product is therefore provided herein that comprises a probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

A milk product is also provided herein that comprises a postbiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*.

A milk product is also provided herein that comprises a postbiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising metabolites from at least one *Clostridium* species selected from the group consisting of: a *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

A milk product may also be a prebiotic composition as described herein.

In one example the probiotic and/or postbiotic compositions provided herein reduce pathogen overgrowth in the gut, promote gut development, promote gut maturation, promote immunity, decrease gut inflammation, and/or promote enteral feeding tolerance in the subject.

Improving the gut microbiome and/or gut health of a subject

The compositions provided herein are for improving the gut microbiome and/or gut health of a subject. In this context, the terms "improving" or "improve" refer to altering the bacterial composition of the gut microbiome in a beneficial way or altering the gut health of the subject in a beneficial way. In the context of improving the gut microbiome, it refers to altering the relative abundance of a variety of bacterial species in the gut microbiome of the subject in a beneficial manner (e.g. to move from a state of dysbiosis of the gut microbiome towards a state of eubiosis). Eubiosis of the gut microbiome refers to the healthy and balanced state of bacterial populations within the gut. Dysbiosis of the gut microbiome is a negative alteration in the relative abundance of bacterial species in the gut and is associated with disease. Dysbiosis may also be referred to as a disturbance of eubiosis. Dysbiosis of the gut microbiome can be caused by infections, inflammation, diet, xenobiotics (including antibiotics, drugs, food additives), and birth route. Methods of determining whether the gut microbiome has been improved, and identifying eubiosis and dysbiosis of the gut microbiome are well known. For example, see US2022/0313759, which provides several assays for determining an improvement in the gut microbiome, which are incorporated herein in their entirety. Methods for improving the gut microbiome and/or gut health of a subject are also well known and include consumption of exogeneous prebiotics, probiotics and/or postbiotics.

Improvement of the gut microbiome and/or improving gut health can provide one or more beneficial effects. For example, it may reduce pathogen overgrowth in the gut, promote gut development, promote gut maturation, promote immunity, decrease gut inflammation, and/or the promote enteral feeding tolerance in the subject. Each of these beneficial effects can readily be identified by a person of skill in the art, for example by comparing one or more of pathogen overgrowth, gut development, gut maturation, gut inflammation, enteral feeding tolerance etc in the subject before and after compositions of the invention have been consumed by the subject for an appropriate length of time. Appropriate consumption regimens for probiotics to have a beneficial effect are known in the art.

Improvement of the gut microbiome (as well as the associated health benefits described above) can in turn lead to subsequent improvements for the subject such as an improved eye sight (e.g., an improvement in retinopathy of prematurity (ROP)) or improved respiratory tract function (e.g. improved respiratory tract function in subjects with Bronchopulmonary dysplasia (BPD)). Enrichment of *Enterobacteriaceae* in the gut microbiome of preterm infants has been

associated with ROP (Skondra et al., J AAPOS. 2020 Aug; 24(4): 236–238) and different probiotics have been shown to reduce the relative abundance of species from within this family (e.g., *Klebsiella*) (Beck at al., *Nature Microbiology* volume 7, pages 1525–1535 (2022)). Enrichment of *Enterobacteriaceae* and reduced diversity in the gut microbiome of preterm infants has also been associated with BPD (Chen et al., m J Perinatol 2021; 38(11): 1142-1149).

Accordingly, the compositions provided herein may be of benefit to subjects with retinopathy of prematurity (ROP) and Bronchopulmonary dysplasia (BPD)). In such cases, the composition may be used as a supplement by these subjects, to improve the symptoms and signs or disease.

The compositions described herein may also be used to reduce the relative abundance of one or more pathobionts in the gut of a subject. For example, the compositions may be used to inhibit the growth of one or more pathobionts in the gut of a subject. The compositions may be used to inhibit the growth of e.g. one or more of *E. coli*, *E. faecalis*, *K. oxytoca* and/or *K. pneumoniae.* This is particularly applicable to the postbiotic compositions of the invention, as shown in the examples section below.

The compositions described herein may also be used to reduce *Enterobacteriaceae* relative abundance.

The compositions described herein may also be used in the treatment or prevention of a gastrointestinal disorder, allergy, nappy rash, colic, or a symptom thereof in a subject.

The term “gastrointestinal disorder” as used herein refers to disorders and diseases of the digestive system (namely the oesophagus, stomach, small intestine, large intestine, and/or rectum) or accessory organs (such as the liver, pancreas, and gallbladder). It also refers to disorders that originate from problems with the digestive system. A subject with a gastrointestinal disorder will commonly experience symptoms including, but not limited to, diarrhoea, abdominal pain, gastrointestinal bleeding, and/or intestinal obstruction. A gastrointestinal disorder can be either a functional gastrointestinal disorder or an organic gastrointestinal disorder. A functional gastrointestinal disorder is not associated with any physiological changes. Non-limiting examples of functional gastrointestinal disorders include irritable bowel syndrome (IBS), gastroesophageal reflux disease, functional dyspepsia, functional nausea and vomiting, functional abdominal pain, functional constipation, and faecal incontinence.

An organic gastrointestinal disorder has measurable physiological changes and often results from intestinal inflammation. Non-limiting examples of organic gastrointestinal disorders

include Crohn's disease, ulcerative colitis, coeliac disease, inflammatory bowel disease (IBD), chronic gastrointestinal infections, microscopic colitis, intestinal inflammation, colitis, necrotising enterocolitis (NEC), late-onset sepsis, and focal intestinal perforation.

The compositions described herein may also be used in the treatment or prevention of a gastrointestinal disorder, selected from the group consisting of: intestinal inflammation, irritable bowel syndrome, colitis, necrotising enterocolitis, focal intestinal perforation, late-onset sepsis, and inflammatory bowel disease.

The term "intestinal inflammation" as used herein covers both acute and chronic inflammation of the tissues in a subject's digestive tract, and is classified by redness (rubor), swelling (tumour), pain (dolor), and/or loss of function (functio laesa) as a result of the subject's body's reaction against injury and infection orchestrated by the immune system.

The term "irritable bowel syndrome" as used herein refers to a group of symptoms that occur together, including, but not limited to, recurrent stomach cramps, bloating, distension, diarrhoea, and/or constipation.

The term "colitis" as used herein refers to inflammation of the lining of the large intestine (colon). Any disease or disorder which results in acute or chronic inflammation of the large intestine falls under this term. Non-limiting causes of colitis include infections caused by viruses, parasites or bacteria, Crohn's disease, ulcerative colitis, necrotising enterocolitis. Colitis commonly manifests as abdominal pain and bloating, bloody stools, tenesmus, dehydration, diarrhoea, and/or fever.

The term "necrotising enterocolitis (NEC)" as used herein refers to the acute inflammatory disorder responsible for necrosis of the intestinal tissue. NEC predominantly develops in preterm infants and rarely affects infants born at term or adults. Infants usually develop it within two to six weeks after birth. NEC can be mild or severe, with the latter experiencing life-threatening symptoms.

The term "intestinal perforation" as used herein refers to the loss of continuity of the gastrointestinal or bowel wall and is a common complication of a number of diseases or disorders. Non-limiting examples resulting in intestinal perforation comprise appendicitis, cancers affecting the digesting system, cancers of the digestive system, diverticulitis, hernias, bowel obstruction, gall stones, colitis, trauma, ischaemia, inflammation, and necrotising enterocolitis. In the context of newborns and infants, a specific example is focal intestinal perforation (FIP), which is a single intestinal perforation that is typically found at the terminal ileum.

The term “sepsis” is defined as the dysregulated host response to infection encompassing a spectrum of disease/disorder, ranging from minor signs and symptoms to life-threatening organ dysfunction and death. Infections that lead to sepsis most often start in the lung, urinary tract, skin, or gastrointestinal tract. Sepsis may progress to septic shock, which comprises a dramatic drop in blood pressure which can damage vital organs and can be fatal. The term “early-onset sepsis” as used herein refers to sepsis onset most often appearing within 24 to 48 hours of birth, and has commonly contracted the infection from the mother before or during delivery. The term “late-onset sepsis” as used herein refers to sepsis onset after 72 hours and up to 90 days of life and is the pathogens responsible are usually acquired from the environment during or after delivery. It is more common in preterm infants.

The term “inflammatory bowel disease” (IBD) is a term used to describe two conditions: ulcerative colitis and Crohn’s disease. Ulcerative colitis and Crohn's disease are long-term conditions that involve inflammation of the gut. Ulcerative colitis only affects the colon (large intestine). Crohn's disease can affect any part of the digestive system, from the mouth to the anus. Symptoms of IBD include pain, cramps or swelling in the tummy; recurring or bloody diarrhoea; weight loss and extreme tiredness.

The compositions provided herein result in an improvement of the gut microbiome and/or gut health of a subject, which can provide one or more beneficial effects. For example, it may reduce pathogen overgrowth in the gut, promote gut development, promote gut maturation, promote immunity, decrease gut inflammation, and/or the promote enteral feeding tolerance in the subject.

The terms “small intestinal bacterial overgrowth”, “SIBO”, and “pathogen overgrowth” as used herein are interchangeable and refer to the presence of excess colonic bacteria in the small intestine resulting in an unbalanced microbiota. Commonly present in SIBO are *Escherichia coli*, *Aeromonas* species, and *Klebsiella* species, but *Bacteroides, Clostridium, Peptostreptococcus,* and *Micrococcus* species are also responsible.

The term “gut development” as used herein refers to the process of establishing the microbiota from birth. A similar term “gut maturation” as used herein is intended to refer to the process of the maturing of the microbiota composition to an adult-like composition and function between three and five years of age.

The term “immunity” as used herein broadly refers to the subject’s state of being insusceptible or resistant to a pathogen or infectious disease. Immunity is governed by the immune system, a system of defence comprising a complex network of organs, cells, tissues, and other components, such as proteins, which act together to help protect the subject. The three types of immunity are innate, adaptive, and passive. Innate immunity is the system with which you

were born with and is characterised by being non-specific. Non-limiting examples of innate immunity comprise stomach acid, the skin barrier, mucus, and enzymes in saliva and skin oils. Adaptive immunity is the system conferring acquired immune responses or 'active' immunity, resulting in immunological memory after an initial response to, usually, a pathogen. Non-limiting examples of adaptive immunity comprise an immature B cell becoming a plasma cell and producing specific antibodies to an epitope on an antigen. Passive immunity is immunity provided by the administering of exogenous immune components (either naturally or artificially) which provide short-term protection to the subject. Non-limiting examples of passive immunity comprise a baby receiving its mother's antibodies through the placenta or breast milk (natural passive immunity) or the administering of antibodies to neutralise a toxin (artificial passive immunity).

The term "enteral feeding" as used herein refers to nutrition taken through the mouth or through a tube that goes directly to the stomach or small intestine. The latter meaning is often used in a medical setting. In turn, the term "enteral feeding tolerance" as used herein is intended to refer to the absence of an adverse reaction to the delivery of nutrition or food taken through the mouth or through a tube that goes directly to the stomach or small intestine.

The compositions provided herein may also be used to treat or prevent an allergy in a subject. The term "allergy" as used herein refers to a subject's immune system's hypersensitive response to a foreign, but inert, substance (i.e., an allergen) in the environment. Allergies can occur on a range of severity, from a blocked nose to anaphylaxis. Allergic reactions can be mediated by IgE or be non-IgE in nature. Non-limiting examples of allergens can include food, insect stings, latex, and drugs. Non-limiting examples of food allergens in allergenic food include celery, gluten containing cereal (such as barley, wheat, and oats), crustaceans (such as crabs, prawns, and lobsters), fish, milk, molluscs (such as mussels and oysters), eggs, mustard, peanuts, sesame, soybeans, sulphur dioxide and sulphites and tree nuts (such as hazelnuts, walnuts, almonds, cashews, pecans, macadamia nuts, pistachio, and brazil nuts).

A further example of an allergy is cows' milk allergy (CMA), also called cows' milk protein allergy, is one of the most common childhood food allergies. CMA typically develops when cows' milk is first introduced into the baby's diet either in formula or when your baby starts eating solids. More rarely, it can affect babies who are exclusively breastfed because of cows' milk from the mother's diet passing to the baby through breast milk. There are 2 main types of CMA:

- immediate CMA – where symptoms typically begin within minutes of having cows' milk
- delayed CMA – where symptoms typically begin several hours, or even days, after having cows' milk.

Cows' milk allergy can cause a wide range of symptoms, including:

- skin reactions – such as an itchy rash or swelling of the lips, face and around the eyes
- digestive problems – such as stomach ache, vomiting, colic, diarrhoea or constipation
- hay fever-like symptoms – such as a runny or blocked nose
- eczema that does not improve with treatment

Occasionally CMA can cause severe allergic symptoms that come on suddenly, such as swelling in the mouth or throat, wheezing, cough, shortness of breath, and difficult, noisy breathing.

The compositions provided herein may also be used to treat or prevent nappy rash in a subject. The term “nappy rash” as used herein refers to the acute inflammatory reaction of the skin in contact with a nappy. It can be characterised by inflamed, irritated, red, and/or dry skin in contact with the nappy and is primarily characterised as an irritant contact dermatitis. It is commonly caused by the prolonged skin contact with the subject’s urine or faeces, skincare preparations, or detergents and fabric softeners.

The compositions provided herein may also be used to treat or prevent an colic in a subject. The terms “colic” and “infantile colic” as used herein are interchangeable and refer to the paroxysms of crying of a healthy infant when there is no obvious cause for the infant to be crying. Colic is a pattern of crying, which occurs daily and often at the same time of day which occurs in up to a quarter of newborn babies. It is most common during the first six weeks of life and will usually resolve by four to six months independent of therapeutic intervention.

The compositions provided herein may reduce the proportion of pathogenic bacteria in the microbiome of the subejct. In some embodiments, the pathogenic bacteria are *Enterobacteriaceae* (e.g., one or more of *Salmonella, E. coli,* or *Klebsiella*). In some examples, the pathogenic bacteria are reduced by greater than 10%, 15%, 25%, 50%, 75%, 80%, or 85% by the treatment.

The compositions provided herein may reduce the antibiotic resistance gene load in the subject. One or more genes of the antibiotic resistance gene load may be reduced by greater than 10%, 15%, 25%, 30%, 45%, 50%, 75% or 85%. In some examples, the compositions provided herein may reduce the levels of lipopolysaccharide (LPS) and/or pathogenic bacteria in the gut of the subject.

The compositions provided herein may reduce the frequency of bowel movements in the subject as compared to a dysbiotic subject. In some examples, the stool composition of an subject be altered as compared to a dysbiotic subject. The firmness/consistency of the stool

composition of the subject can be increased as compared to a dysbiotic subject. In some examples, the stool can be less watery.

Subjects

The compositions described herein may be of benefit to any appropriate subject. The term "subject" as used herein is intended to include humans and animals. The terms "subject", "individual", and "patient" are used herein interchangeably. Examples of subjects include mammals, e.g., humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In some examples, subjects include companion animals, e.g. dogs, cats, rabbits, and rats. In some examples, subjects include livestock, e.g., cows, pigs, sheep, goats, and rabbits. In some examples, subjects include thoroughbred or show animals, e.g. horses, pigs, cows, and rabbits. The subject may be an infant.

In some examples, the subject is a human. The subject may be a human infant. The term "human infant" as used herein is intended to refer to a child in the first year of life.

The subject may be less than four weeks old, which may be referred to herein as "neonate".

The subject may be born at term or preterm. The subject may be born alive before 37 weeks of pregnancy, which may be referred to herein as a "preterm infant".

The subject may be an infant who is small for their gestational age, which comprises an infant born with a birth weight less than the 10th centile (based on age).

The subject may be an infant born by Caesarean-section (C-section).

In some examples, the subject may be an infant prior to the NEC risk period. In some examples, the subject may be a newborn during the NEC risk period. In some examples, a subject may be a newborn post-NEC risk period. In this context, the NEC risk period may be defined as the first 10 to 40 days of life.

In some examples, the subject may be an infant that is in (staying in) an NICU (neonatal intensive care unit). In some examples, the subject may be an infant that has been discharged from NICU within the last month.

In some examples, the subject may be 0-3 months old.

In some examples, a subject may be 3-12 months old.

In some examples, a subject may be from 1-5 years old.

In some examples, the subject may be 5-13 years old.

In some examples, the subject may be 13+ years old.

In some examples the subject may be an adult (18+).

In some examples the subject may be elderly (65+).

The subject may be an infant that has been hospitalised and/or is undergoing antibiotic treatment. Infants, including preterm infants on NICU, spending prolonged time in hospital will get colonised by higher levels of hospital acquired pathobiont bacteria and therefore in particular require a stabilization and/or improvement of the gut microbiome.

In any examples described herein, the subject can be dysbiotic. "Dysbiotic" refers to the condition of dysbiosis (also called dysbacteriosis) and is characterized by a disruption to the microbiome resulting in an imbalance in the microbiota, changes in their functional composition and metabolic activities, or a shift in their local distribution. Dysbiosis can refer to any flora such as the skin flora, gut flora, or vaginal flora.

In particular, the subject can be dysbiotic with regard to their gut microbiome. Examples of such subjects may be those that are undergoing antibiotic treatment.

Dysbiosis in a subject, especially an infant, can be observed by the physical symptoms of the subject (e.g., diarrhea, digestive discomfort, inflammation, etc.) and/or by observation of the presence of free sugar monomers in the feces of the subject, an absence or reduction in specific Bifidobacteria populations, and/or the overall reduction in measured SCFA; more specifically, acetate and lactate. Additionally, the infant may have an increased likelihood of becoming dysbiotic based on the circumstances in the environment surrounding them (e.g., an outbreak of disease in the surroundings of the subject, formula feeding, cesarean birth, etc.). Dysbiosis in an infant can further be revealed by a low level of SCFA in the feces of said infant.

As an example, a dysbiotic infant may have (a) a watery stool, (b) *Clostridium difficile* levels of greater than 10^6 cfu/g feces, greater than 10^7 cfu/g feces, or greater than 10^8 cfu/g feces, (c) *Enterobacteriaceae* at levels of greater than greater than 10^6, greater than 10^7, or greater than 10^8 cfu/g feces, and/or (d) a stool pH of 5.5 or above, 6.0 or above, or 6.5 or above.

As used herein, the terms "gut microbiome", "gut flora", "gastrointestinal flora", "intestinal flora", "intestinal microbiome", "microbiota", and "microbiome" are interchangeable and are intended to represent the bacterial community present in the digestive tract of the subject. It is meant to reflect the variety of commensal and mutualistic species, and relative abundance of said bacterial species found in a healthy subject. It is the gut microbiota which functions to ferment non-digestible substrates like dietary fibres and endogenous intestinal mucus (prebiotics), metabolise nutrients, xenobiotic and drugs, maintain structural integrity of the mucosal barrier, immunomodulation, and confer pathogenic resistance.

Methods of administration

Typically, the compositions provided herein are for oral administration. However, other suitable routes of administration of the composition described herein may be via suppositories or feeding tubes directly into the digestive tract, including but not limited to, a jejunostomy tube, gastrostomy tube, or a nasojejunal tube.

The compositions provided herein are intended as a supplement and may be administered regularly for effects to be maintained or persist. After administration, for example by ingestion, probiotics typically adhere to a tissue of the host, such as the digestive tract. Once attached, the desirable bacteria are capable of multiplying and colonising, thereby altering the microbiota of a subject.

Probiotics can be mixed with various ingredients to create a product for consumption.

The compositions provided herein are for administration to the subject at an appropriate (e.g. therapeutically effective) dose. Appropriate doses are readily identifiable by a person of skill in the art.

In one example the probiotic composition provided herein comprises at least 0.5×10^9 CFU of the *Clostridium* species per dose. As would be clear, for compositions that comprise more than one *Clostridium* species, the probiotic composition provided herein may comprise at least 0.5×10^9 CFU of each *Clostridium* species per dose.

The term “dose” as used herein refers to the quantity of the *Clostridium* species administered or taken with one total serving, i.e. it resembles the total intake independent of the volume administered or taken, with one portion. Thereby “one portion” as used herein refers to the recommended amount of the composition independent of the form. It may be a certain amount of tablets, pills, capsules or a certain amount of a (pre-packed) syrup, (pre-packed) powder, (pre-packed) solid beverage, (pre-packed) soup, or (pre-packed) liquid suspension.

The term CFU (colony forming unit) as used herein refers to a single, viable propagule that produces a single colony (a population of the cells visible to the naked eye) on an appropriate semisolid growth medium. Methods for determining the number of CFUs are well known in the art.

In one example the composition comprises at least 0.6×10^9 CFU per dose per strain, at least 0.6×10^9 CFU per dose per strain, at least 1×10^9 CFU per dose per strain, at least 2×10^9 CFU per dose per strain, at least 3×10^9 CFU per dose per strain, at least 4×10^9 CFU per dose per strain, at least 5×10^9 CFU per dose per strain, at least 6×10^9 CFU per dose per strain, at least 7×10^9 CFU per dose per strain, or at least 8×10^9 CFU per dose per strain.

The CFU per dose can vary between the two or three species or can be the same.

In some examples, the composition is provided to the subject on a daily basis comprising from 0.1 billion to 500 billion cfu of bacteria/day. For example, the composition that is provided on a daily basis can include from 1 billion to 100 billion cfu/day or from 5 billion to 20 billion cfu/day. The composition may be provided on a daily basis for at least 2, at least 5, at least 10, at least 20, or at least 30 days. The recipient of the treatment can be a human infant.

Based on the subject's condition the skilled person would be able to choose the right dosage for each strain.

The composition can be administrated orally or by other suitable routes. When administered orally the composition may be in the form of capsules, tablets, powders, granules, solutions, or suspensions etc. The *Clostridium* species can be mixed with conventional excipients, such as gelatin, starch, lactose, magnesium stearate, talc, gum arabic and the like. It may also be advantageous to use less conventional excipients that, for example, make it possible to increase the ability of *Clostridium* species to be active in the gut. For example, cellobiose, maltose, mannose, salicine, trehalose, amygdalin, arabinose, melobiose, rhamnose and/or xylose may be added. This list is not exhaustive and the substrates may be chosen or changed depending on circumstances. Additionally, a capsule, pill, tablet, or syrup for oral administration should be stored in a manner so as to preserve its efficacy. Methods of storage for probiotic compositions include but are not limited to refrigeration, freezing, and storing at room temperature. If stored at room temperature, the *Clostridium* species or compositions thereof should be stored in an air tight container.

The probiotic *Clostridium* species should arrive intactly in the small and large intestine the latter of which may be colonized. The *Clostridium* species may be microencapsulated or enterically coated to provide a release profile that targets replacement or alteration of live *Clostridium* species at a pre-determined location within the gastrointestinal tract of a subject. Such microencapsulation formulations and techniques may protect the live *Clostridium* species from the digestive actions of the stomach, duodenum, and jejunum of the intestine and allow administration, delivery or release to the gut or ileum of a subject. Microencapsulated live *Clostridium* species and compositions thereof may be in various dosage forms, and they can be co-administered with drugs, foods, nutrients, vitamins, other beneficial substances, prebiotics, and other therapeutic agents such as pH encapsulated glucose, lipids or proteins that release in the distal small intestine at pH values between 7.0 and 8.0 in an amount sufficient to achieve the desired effect in a subject. For example, at least two coatings may be used to cover a tablet or capsule like form comprising the *Clostridium* species, wherein the outside coating is degraded in a pH environment of 5 to 6 and the inside

coating is degraded in a pH environment of about 7 thereby releasing the *Clostridium* species in the ileum area. An exemplary coating may include one or more of poly(dl-lactide-co-glycolide) chitosan, casein, chitosan (Chi) stabilized with PVA (poly-vinylic alcohol), a lipid, an alginate, carboxymethylethylcellulose (CMEC), cellulose acetate trimellitiate (CAT), hydroxypropylmethyl cellulose phthalate (HPMCP), hydroxypropylmethyl cellulose, ethyl cellulose, color con, food glaze and mixtures of hydroxypropylmethyl cellulose and ethyl cellulose, polyvinyl acetate phthalate (PVAP), cellulose acetate phthalate (CAP), shellac, copolymers of methacrylic acid and ethyl acrylate, and copolymers of methacrylic acid and ethyl acrylate to which a monomer of methylacrylate has been added during polymerization, and other methacrylic resins that are commercially available under the tradename Eudragit® (Rohm Pharma; Westerstadt, Germany), including Eudragit® L30D-55 and L100-55 (soluble at pH 5.5 and above), Eudragit® L-100 (soluble at pH 6.0 and above), Eudragit® S (soluble at pH 7.0 and above, as a result of a higher degree of esterification), and Eudragits® NE, RL and RS (water-insoluble polymers having different degrees of permeability and expandability); vinyl polymers and copolymers such as polyvinyl pyrrolidone, vinyl acetate, vinylacetate phthalate, vinylacetate crotonic acid copolymer, and ethylene-vinyl acetate copolymer; enzymatically degradable polymers such as azo polymers, pectin, amylose and guar gum; and zein. Combinations of different coating materials may also be used. Multi-layer coatings using different polymers may also be applied. The preferred coating weights for particular coating materials may be readily determined by those skilled in the art by evaluating individual release profiles for tablets, beads and granules prepared with different quantities of various coating materials. It is the combination of materials, method and form of application that produce the desired release.

The compositions described herein may be administered once or more per day to a subject. The administration amount can vary depending on the body weight of the subject, the severity of a subject's condition, the subject's desired outcomes, supplemental active ingredients included and microorganisms used therein. In addition, it is possible to divide up a daily administration amount and to administer continuously, if needed. Suitable dosages of *Clostridium* species or compositions thereof may be provided orally at a dosage rate of at least 10 milligrams to at least 1000 milligrams per day. The dosage rate, effective as a food supplement and for establishing or increasing *Clostridium* species in the intestinal tract of a subject may be determined by known methods in the art.

In some examples, *Clostridium* species or compositions thereof may be administered 1, 2 3, 4, 5 or more times per week for a specified time period. For example, the specified time period may be at least one week. In some examples, the specified time period may be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8 weeks or more. In some

examples, administration may be continuous, for example a subject may continually take doses of *Clostridium* species or compositions thereof even after effects the beneficial effects have been achieved. In the context of medical conditions and diseases that may reduce muscle mass and/or strength, continuous administration may help prevent or reduce the muscle wasting effects of the condition or disease.

An "effective amount" refers to an amount of a *Clostridium* species sufficient to achieve the desired function or activity in the subject and is acceptable to the subject (i.e. does not cause adverse effects in the subject). For example, an effective amount may be an amount of *Clostridium* species that provides some improvement or benefit to a subject having a disease, condition, or disorder in which a loss of muscle mass and/or muscle strength occurs. Thus, an "effective amount" or "therapeutically effective amount" with reference to therapeutic uses and methods is an amount that provides some alleviation, mitigation, and/or decrease in at least one clinical symptom of the disease, condition, or disorder in which a loss of muscle mass and/or muscle strength occurs. Those skilled in the art will appreciate that therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

The effective amount of *Clostridium* species and/or the metabolite thereof in the preparation of the compositions described herein can vary depending on the mode of administration and the requirements of the subject. For example, the severity of a subject's condition or the subject's desired result.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of them mean "including but not limited to", and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. For example, Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY (1994); and Hale and Marham, The Harper

Collins Dictionary of Biology, Harper Perennial, NY (1991) provide those of skill in the art with a general dictionary of many of the terms used in the invention. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the Specification as a whole.

Aspects of the invention are demonstrated by the following non-limiting examples.

Examples

Example I

Experimental Design

To identify novel bacteria that can use HMOs as growth substrates, a feature widely reported to be associated with potentially beneficial bacteria (referred to as probiotic), stool samples were collected from preterm infants in the neonatal intensive care unit at Newcastle, UK. Preterm infant stool samples underwent microbiological culture to isolate a range of novel bacterial isolates. Growth curves were then performed to determine which isolates were capable of growing on specific individual HMOs as the sole carbon source.

Methods

Stool sample collection

Stool samples were regularly collected from nappies/diapers of preterm infants into sterile collection pots by nurse staff. Samples were initially stored at -20°C before being transferred to -80°C for long term storage.

Bacterial isolation and identification

Stool samples were thawed on ice and initially diluted roughly 1:10 w/v in sterile anaerobic PBS. 10-fold serial dilutions were then performed using sterile anaerobic PBS and various dilutions (typically 10^{-2} and 10^{-4}) were cultured by adding 100µl of inoculum onto an agar plate, before spreading to cover the surface of the agar plate and incubating for up to 96 hours. Numerous different agar media was used including brain heart infusion (BHI), Transoligosaccharide propionate (TOS), Bifidus Selective Medium (BSM), De Man, Rogosa and Sharpe (MRS), fastidious anaerobe agar (FAA), and yeast extract-peptone-dextrose (YPD). Unique appearing colonies based on morphology, colour, and size were sub-cultured twice before undergoing full-length 16S sequencing and/or MALDI-TOF to identify the isolate to genus or species level. Isolates were added to glycerol for long term storage.

HMO growth curves

Glycerol stocks were part-thawed and transferred to 5ml broth based on the isolate's preferred medium. Zhang Mills Block 1 (ZMB1) without glucose was prepared, and various carbon sources were tested in 1% concentration. Controls consisted of no carbon source, glucose, and lactose. Tested HMOs were 2'FL, DSLNT, LNT, LNnT, LNFP I, 6SL. The overnight growth for each isolate was centrifuged at 5000g for 5 mins at 4°C, and the pellet was resuspended in the same amount of anaerobic PBS. 20µl of the bacteria in PBS was aliquoted into 180µl of media, and the growth was measured in a Cerillo Stratus plate reader for 150 hours. All isolates were tested in triplicate, and wells with media only were included in each plate to check for contamination.

Results

Only *Clostridium* species and *Bifidobacterium* species were able to grow on HMOs. Within *Clostridium*, the following species were found to use at least one HMO, which has not previously been reported: *C. perfringens*, *C. baratii*, *C. tertium* and *C. paraputrificum* (Fig. 1). Within *Bifidobacterium*, the following species were found to use at least one HMO, which have all been reported previously: *B. infantis*, *B. bifidum*, *B. breve*, and *B. longum* (Fig. 1). The dendrogram shows that 4 strains of *C. perfringens* cluster closely with a *B. infantis* strain. A further 3 strains of *C. perfringens* and a strain of *C. tertium* also form a larger cluster with *B. infantis* from the next branch up. Another cluster is comprised of a *C. tertium*, *C. paraputrificum* and *C. baratii*, which have grouped with 3 strains of *B. breve* and a *B. longum*. The final HMO-utilising cluster formed is comprised of another *C. paraputrificum* alongside an additional *B. breve*. Notably, due to limited DLSNT, not all strains of *Clostridium* could be tested for growth on this HMO. Of the three *Clostridium* strains tested on DLSNT, two *C. perfringens* were able to use this HMO. Of seven *Bifidobacterium* strains tested on DLSNT, *B. bifidum* and a single *B. breve* showed growth. Growth of *B. bifidum* on DSLNT was higher than that seen for both *C. perfringens* strains and the growth of *B. breve* was either lower or comparable to them. Of all *Bifidobacterium* strains, only *B. animalis* was not tested on DLSNT and it is unlikely this strain can use this HMO given it could not use any other HMO or lactose for growth. Aside from DLSNT, all *C. perfringens, C. baratii*, and *C. tertium*, and one *C. paraputrificum* strain were able to use LNnT. *C. baratii*, *C. tertium*, both *C. paraputrificum* and one other strain of *C. perfringens* could use LNT. Most *C. perfringens* strains could use 6SL (6/7) and 2FL (4/7). Only one stain of *C. perfringens*, one strain of *B. infantis*, and one strain of *B. bifidum* could use LNFP_I. When growing on HMOs, HMO-utilising bacteria produce short chain fatty acids, convert aromatic amino acids into aromatic lactic acids (e.g., indolelactic acid; ILA), and potentially release other functionally important compounds that have important

immunomodulatory roles in host health. (Laursen et al. Nat Microbiol. 2021; Henrick et al. Cell. 2021 Jul 22;184(15)). For instance, ILA has been shown to limit T cell activation and reduce a pro-inflammatory response by inducing galectin-1 in T_H2 and T_H17 cells during helper T cell polarisation (Henrick et al. Cell. 2021 Jul 22;184(15)). In terms of short chain fatty acid production, *Clostridium* are able to produce butyrate when from dietary glycans and higher butyrate has previously been linked to reduced NEC risk. Thus, HMO-utilising bacteria are widely considered probiotic bacteria and higher levels of HMO-utilising bacteria in the gut has been associated with reduced inflammation and improved health. Furthermore, higher levels of DLSNT in mothers own milk is linked to reduced risk of NEC, potentially relating to modification of the infant gut microbiome and promotion of bacteria that can utilise this HMO. The disclosure provides a method for increasing *Clostridium* abundance in the gut, including strains that are able to use a range of HMOs including DSLNT.

Example II

Experimental Design

To identify if HMO-utilising bacteria found in the preterm infant gut microbiome, namely *Clostridium* species and *Bifidobacterium* species, are associated with protection from NEC, preterm infants diagnosed with NEC provided longitudinal stool samples spanning the disease course. To identify bacteria enriched in non-NEC preterm infants, healthy controls who did not develop NEC also provided longitudinal stool samples.

Methods

Stool sample collection

As previously explained, stool samples were regularly collected from nappies/diapers of preterm infants into sterile collection pots by nurse staff. Samples were initially stored at -20°C before being transferred to -80°C for long term storage.

Metagenomic sequencing

DNA was extracted from 100mg of stool sample using the DNeasy PowerSoil Kit (QIAGEN). Library prep was performed using the Nextera DNA Flex Kit. Sequencing was performed on the HiSeq X Ten (Illumina) with a target read depth of 10M reads per sample with a read length of 150bp paired end reads. Taxonomic profiling of metagenomic samples was performed using MetaPhlAn v.2.0. All samples containing <100,000 were removed from the analysis.

Analysis of metagenomic dataset

The relative abundance of the six most abundant *Bifidobacterium* species and six most abundant *Clostridium* species were first plotted over the first 150 days of life, faceted by NEC or healthy status. Correlations were visually inspected, with particular focus on the critical period of NEC risk between days 10 and 40 when most NEC is diagnosed. Generalized linear mixed models (GLMMs) were fit to the data using the glmmTMB package v.1.0.2.1 in R. The general formula for each of the LMMs fitted was as follows:

$Y \approx X_1 + X_2 + \ldots + X_n + (1|\text{SubjectID})$.

To find out which co-variates were significantly associated with *Clostridium perfringens* relative abundance, mixed-effects models were fit using the Gaussian distribution. NEC status, breastmilk status, and birth mode were included as fixed effects and subject ID was included as a random group intercept.

Results

Clostridium perfringens was elevated early in life in healthy (no-NEC) preterm infants in comparison to preterm infants diagnosed with NEC (Fig. 2). This was especially apparent over the critical period of NEC risk between days 10 and 40 when most NEC is diagnosed (Fig. 2). In contrast, the six most abundant *Bifidobacterium* species showed relatively similar trajectories between healthy and NEC diagnosed preterm infants (Fig. 3). GLMMs of *Clostridium perfringens* before (preNEC) and after (postNEC) NEC diagnosis compared to healthy preterm infants as the reference group show a significant increase in *C. perfringens* in healthy preterm infants prior to disease (Fig. 4). No difference was shown in *C. perfringens* relative abundance in preterm infants after they recover from NEC compared to healthy preterm infants (Fig. 4). This disclosure provides a method of increasing *Clostridium* abundance, including but not limited to *Clostridium perfringens*, in the (preterm) gut to reduce the risk of NEC and other diseases.

Example III

Experimental Design

To identify what effect sterile postbiotics derived from *C. perfringens* and *C. tertium* would exert on the growth of other microbes isolated from the neonatal or adult gut, specifically whether they would inhibit the growth of pathobionts and promote the growth of *Bifidobacterium*.

Methods

Stool sample collection

As previously explained, stool samples were regularly collected from nappies/diapers of preterm infants into sterile collection pots by nurse staff. Samples were initially stored at -20°C before being transferred to -80°C for long term storage.

Preparation of postbiotic

A loopful of a glycerol stock was streaked out across an agar plate of the isolate's preferred medium and incubated anaerobically at 37°C overnight. A single colony was transferred to 5ml broth based on the isolate's preferred medium and incubated anaerobically at 37°C overnight. Two 5 mL aliquots of ZMB1 were prepared. Glucose (5 mg/ml) was added one aliquot, while the HMOs (5 mg/ml of each) known to sustain growth of the isolate were added to the second aliquot. The OD600 of the overnight growth of the isolate was measured and the culture diluted to OD 1. Two 250 µL aliquots of the culture were then centrifuged at 5000g for 5 mins at 4°C, and the pellet was resuspended in the same amount of anaerobic PBS. Centrifugation was repeated and the pellets then resuspended in 250 µL of 'ZMB1 with glucose' or 'ZMB1 with HMOs' respectively. The culture aliquots were then added to the remaining 4.75 mL of 'ZMB1 with glucose' or 'ZMB1 with HMOs'. The two cultures were then incubated anaerobically, at 37°C, with shaking at 130 rpm, for 2-6 days. At the end of the incubation period, the two cultures were centrifuged at 5000g for 5-10 mins at 4°C, and the supernatants transferred to new tubes in the anaerobic chamber. These supernatants were then filter sterilised with Merck Millex™-GP Sterile 0.22 µm syringe filters, producing cell free supernatants (CFSs). ZMB1 is described in detail in Zhang et al Appl Environ Microbiol. 2009 Feb; 75(4): 1080–1087. Other appropriate media may also be used.

Assay to determine activities of postbiotic on different pathobionts and *Bifidobacterium spp.*

Glycerol stocks of either pathobionts or *Bifidobacterium* were streaked out on agar plates of each isolate's preferred medium and incubated anaerobically at 37°C overnight. Single colonies were then picked from these plates, transferred to 5ml broth based on each isolate's preferred medium and incubated anaerobically at 37°C overnight. On the same day, a fresh batch of ZMB1 medium was prepared and split into 4. One of the ZMB1 aliquots was supplemented with glucose to concentration of 10 mg/mL, the second with glucose to a concentration of 20 mg/mL, and the third with the same HMOs used in the postbiotic to a total concentration of 10 mg/mL. The fourth aliquot was left without a carbon source. All media aliquots were then left in the anaerobic chamber overnight to deplete oxygen (i.e. to remove any oxygen). The next day, the OD600 of each pathobiont culture was measured and volumes required to dilute each to an OD600 of 0.5 in 1.5 mL ZMB1 were calculated. The required volumes of the cultures were then centrifuged at 5000g for 5 mins at 4°C and the supernatants

removed. The pellets were then resuspended in 1 mL anaerobic PBS and centrifuged again as before, with the supernatants removed. All pellets were then resuspended in 1.5 mL ZMB1 with 10 mg/ml glucose. The cultures were then incubated anaerobically at 37°C, with shaking at 110 rpm, until they each reach mid exponential growth phase. At this point, the OD600 of each was measured and volumes required to dilute them to an OD600 of 0.1 in 1.5 mL ZMB1 were calculated. The required volumes of the cultures were then centrifuged at 5000 G for 5 mins at 4°C and the supernatants removed. The pellets were then resuspended in 1.5 mL ZMB1 + 20 mg/mL glucose.100 uL of each pathobiont was then added to either 1) 100 uL of ZMB1 with HMOs postbiotic, 2) 100 uL of ZMB1 with glucose postbiotic, 3) 100 uL of sterile ZMB1 with HMOs medium, and 4) 100 uL of sterile ZMB1 medium (no carbon source). Following this set up, the minimum concentration of glucose in each well was 10 mg/mL. All experiments were performed in triplicate. 200 uL of each media condition used (ZMB1, 'ZMB1 with glucose', 'ZMB1 with HMOs' and the two postbiotics) was added to 5 separate wells to act as blanks and to check for contamination during the assay. The plate was then shaken at 100 rpm for 1 minute. The lid was then removed and the plate sealed with a Diversified Biotech Breathe-Easy plate seal. The sealed plate was then placed in a Cerillo Stratus plate reader and the assay run for 24 hours, with readings taken at 600 nm.

Results

For the *C. perfringens* derived HMO postbiotic, growth of five pathobionts tested (*E. coli*, *E. faecium*, *K. oxytoca*, *K. pneumoniae* and *E. cloacae*) was significantly inhibited during the first 10 hours of incubation, compared to the regular medium control (Fig. 5). For the *C. tertium* derived HMO postbiotic, the same inhibition of growth was also observed during the first 10 hours of incubation, but for a wider range of pathobionts (*E. coli*, *E. faecium*, *K. oxytoca*, *K. pneumoniae, E. cloacae*, *E. faecalis* and *S. haemolyticus*) (Fig. 6). This effect was not due to the presence of unmetabolized HMOs, as shown by the 'HMO control' condition having produced similar AUCs to the regular medium control and higher AUCs compared to the HMO postbiotic. For all pathobionts tested, the glucose postbiotics also inhibited growth compared to controls, demonstrating that the inhibitory effect was not solely dependent on HMO metabolism by the probiotic strain.

Both *C. perfringens* and *C. tertium* derived HMO postbiotics promoted the growth of three of the *Bifidobacterium spp.* tested (*B. bifidum, B. breve* and *B. longum*) during 72 hrs of incubation, compared to regular medium controls (Figs. 7 & 8). The *C. perfringens* derived glucose postbiotic also produced similar growth promotion, demonstrating the effect was not solely dependent on HMO metabolism by the probiotic strain. The same was also true for the

activity of the *C. tertium* glucose postbiotic on *B. longum*. However, for *B. bifidum* and *B. breve*, the *C. tertium* HMO postbiotic had a stronger growth promoting effect, suggesting metabolism of HMOs by *C. tertium* may indeed result in the production of additional bifidogenic metabolites compared to metabolism on glucose. *C. perfringens* and *C. tertium* derived HMO and glucose postbiotics all inhibited growth of *B. infantis* compared to regular medium controls (Figs. 7D & 8D). However, this inhibition was weaker than the effect seen against the pathobionts above, as differences in growth between postbiotic and control conditions were not detectable at 10 hours of incubation, but instead only at 72 hours. This suggests the *C. perfringens* and *C. tertium* derived postbiotics would not interfere with initial *B. infantis* colonisation and establishment in the gut environment, as they likely would with the pathobionts tested above.

These results suggest that both *C. perfringens* and *C. tertium* can utilise HMOs and glucose to produce effectors that selectively target and inhibit the growth of pathobionts, while also promoting the growth of health-associated *Bifidobacterium*.

Example IV

Experimental design

To identify whether live *C. perfringens* inhibits the growth of *B. infantis* when the two are incubated together as live cultures.

Methods

Co-culture of *B. infantis* and *C. perfringens*

Bacteria were grown in their preferential media overnight before the co-cultures were set up in ZMB1 supplemented with 1% w/v of a mix of LNT, LNnT, 6SL, 2'FL and LNFP I (each HMO will have a final concentration of 0.2% w/v). A starting total OD of 0.1 was used, and bacteria were cultured as a single culture, or as co-cultures in various proportions, 50%+50%, or 25%+75%. A co-culture made by an equal amount of all bacteria was also tested. The growth was stopped at 24h, and 500ul of culture were harvested at the start and at the end of the experiment. The cultures were centrifuged at 17000G for 5 mins, the supernatant was saved for further analysis, and the DNA was extracted from the pellet using the DNeasy Microbial Kit (Qiagen) following the manufacturer's instructions. The DNA was resuspended in 50ul of elution solution. Quantitative PCR was then used to determine the

numbers of cells for each bacteria using species-specific primers. The specificity of each primer was tested against all the isolates included in the experiments. Each reaction was composed of a total volume of 20µl of which 10µl of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 1µl of template DNA, and forward and reverse primers to achieve the final concentration.

Results

When co-cultured on HMOs with increasing percentages of *C. perfringens*, no significant difference in the growth of *B. infantis* was found between the co-culture conditions and *B. infantis* cultured in isolation (Fig. 9). Therefore, live *C. perfringens* does not appear to inhibit the growth of *B. infantis*.

Example V

Experimental design

To compare the impact of increasing dosages of *Clostridium* derived HMO and glucose postbiotics compared to *Bifidobacterium* derived HMO and glucose postbiotics on viability human cells.

Methods

MTS assay to measure viability of Caco-2 cells incubated with postbiotics

Caco-2 cells were cultured in Advanced DMEM, supplemented with FBS to 10% and GlutaMAX. For the assay, the cells were seeded at a density of 5000 cells per well, in a volume of 200 µL per well, across 96 well plates and incubated at 37°C and 5% CO_2 for 24 hours. The old medium was then removed, and the following conditions set up in 100 µL 1) DMEM only control, 2) ZMB1 medium, with no carbon source at 10%, 25%, 50% and 75% dilutions in DMEM, 3) 11x HMO postbiotics in separate wells at 10%, 25%, 50% and 75% dilutions in DMEM, 4) 12x glucose postbiotics in separate wells at 10%, 25%, 50% and 75% dilutions in DMEM. All conditions were set up in triplicate. The assays were then incubated at 37°C and 5% CO_2 for 24 hours. Media was then removed from each well and replaced with 100 µL fresh DMEM. MTS reagent is reduced by metabolically active cells into a formazan salt that is soluble in tissue culture medium, giving a detectable colour change which can be quantified by measuring absorbance at 490 nm using a plate reader. The

measured absorbance at 490 nm corresponds to the quantity of formazan produced, which in turn corresponds to the number of living cells in the culture. Thus, the measured absorbance is directly proportional to the number of viable cells and allows the viability of a culture to be compared with others. Fresh MTS reagent was prepared by adding phenazine methosulfate (PMS) to a concentration of 5% to MTS tetrazolium salt solution. 20 µL of the reagent was then added to each well of the assay plate, which was then incubated at 37°C and 5% CO_2 for 4 hours. MTS reagent was also added to triplicate wells containing only DMEM, to act as a blank. The absorbance at 490 nm of each well was then measured. Following blank correction, absorbances were converted to % viabilities, with absorbances for DMEM only controls being used as the denominators in % calculations. DMEM only controls were therefore set as 100% viability and % viabilities for postbiotic and ZMB1 conditions were calculated relative to those controls. Calculated viabilities for each condition were then averaged. Statistical comparisons were performed at the genus level, with an ANOVA and Tukey post hoc test used to compare average % viabilities for *Clostridium* and *Bifidobacterium* postbiotics, and blank ZMB1 medium, across each concentration tested.

Results

Caco-2 viability with blank ZMB1 medium only fell below 100% at a concentration of 75% (Fig. 10), most likely due to the over dilution of the DMEM tissue culture medium, which would have deprived the Caco-2 cells of essential nutrients. This dilution effect therefore also explains some of the reduction in viability seen for the postbiotics at 75%. However, across the four concentrations used, addition of all postbiotics resulted in lower viabilities compared to blank medium. Both *Bifidobacterium* and *Clostridium spp,* therefore do produce factors that are toxic to human cells when present in sufficient quantities. At concentrations of 10%, the majority of postbiotics show viabilities greater than or equal to that of the DMEM controls, with 15 at or above 100% viability. At concentrations of 25%, only three postbiotics have caused cell viability to fall to below 50%, while seven have only reduced to between 50 and 80%. The remaining 13 are all above 85%, showing the majority are not exerting a strong toxic effect at this concentration. At a concentration of 50%, 14 of the postbiotics have caused viability to fall to below 50% and none of the remainder have a viability higher than 71%. A majority of the postbiotics therefore have an IC50 that lies in the vicinity of 50%, so toxicity is far stronger at >50% concentration. Statistical tests showed that at each concentration showed no significant difference between the average viability of cells incubated with *Clostridium* derived postbiotics compared to those incubated with widely reported beneficial *Bifidobacterium* derived postbiotics. Furthermore, no differences could be seen between HMO and glucose postbiotics.

These results show that when testing these postbiotics against human cells and tissues in further experiments, a maximum concentration of 25% would be most appropriate, it being roughly half of the IC50 of most of the postbiotics. Furthermore, metabolism of HMOs does not appear to cause *Clostridium* or *Bifidobacterium spp.* to produce additional or fewer toxic factors compared to metabolism on glucose. Finally, *Clostridium* derived postbiotics are no more toxic to human cells than *Bifidobacterium* derived postbiotics.

Example VI

Experimental design

To identify whether *C. perfringens* derived HMO postbiotic can suppress inflammation and improve barrier function in a human intestinal epithelial organoid model. To identify how any observed effects in the organoid model compare to those induced by a *B. infantis* derived HMO postbiotic.

Methods

Establishment and culture of human intestinal organoids

Intestinal epithelial organoid lines were established from intestinal crypts isolated from preterm neonate tissue, as described by Stewart et al. (Methods Mol Biol. 2020;2121:185-198). Organoids were cultured and passaged as described in the same protocol.

Organoid monolayer co-culture with bacterial postbiotics and inflammatory stimuli

Three dimensional organoids were processed into 15 two dimensional monolayers in 6.5 mm Transwells, following the protocol described by Fofanova et al. (bioRxiv 555755, 2019). Monolayers were then placed into the anaerobic co-culture system (Fofanova et al., bioRxiv 555755, 2019), which was in turn placed into an anaerobic chamber. Oxygen is flowed through the base of the co-culture unit, maintaining aerobic conditions on the basolateral sides of the monolayers, while the apical sides remain under the anaerobic conditions of the chamber. Thus, the physiological oxygen gradient of the gut epithelium is recreated in this model. The following conditions were set up in triplicate across the 15 monolayers: 1) No stimuli, no postbiotic ('Control'), 2) + apical postbiotic to 25% ('Postbiotic (25%)'), 3) + apical LPS (100 ng/mL) + basolateral flagellin (100 ng/mL) ('Stimuli'), 4) + apical postbiotic to 25% + apical LPS (100 ng/mL) + basolateral flagellin (100 ng/mL) ('Postbiotic (25%) and Stimuli'),

5) + apical postbiotic to 10% + apical LPS (100 ng/mL) + basolateral flagellin (100 ng/mL) ('Postbiotic (10%) and Stimuli'). The experiment was run for 3 hours. The trans-epithelial resistances (TEERs) of the monolayers were measured with an epithelial Ohm meter before the start and at the end of the experiment. ANOVAs followed by Tukey's Post Hoc tests were used to analyse pre-treatment and post-treatment TEERs as separate groups. Conditions within those groups with matching letters were not significantly different. Furthermore, after the incubation, apical and basolateral supernatants were harvested for cytokine assays.

Cytokine assays

Secreted IL8 was measured using a DuoSet ELISA kit, following the manufacturer's protocol, while all other cytokines were measured using custom U-Plex assays from Meso Scale Discovery Inc, also following the manufacturer's protocol. ANOVA and Tukey's Post Hoc tests were used to perform statistical analysis of cytokine secretion data.

Results

The first organoid co-culture experiment used *C. perfringens* derived HMO postbiotic (Fig. 11). No statistical difference was found between the 'Control', 'Stimuli' and 'Postbiotic (10%) and Stimuli' TEERs. There were statistical differences between the 'Control' and the 'Postbiotic (25%)' and 'Postbiotic (25%) and Stimuli' TEERs. These two 25% postbiotic conditions were also statistically different from the 'Stimuli' condition and induced the first and second highest average changes in TEER (Fig. 11A). The larger increases in TEERs therefore appear to be a result of a biological activity of the postbiotic itself. In other words, in the presence of microbially induced inflammatory insult, the postbiotic was able to increase barrier integrity of the preterm intestinal epithelium, which is a protective characteristic as described below. The *C. perfingens* derived postbiotic was also found to inhibit the secretion of pro-inflammatory cytokines and chemokines from the organoid monolayers (Fig. 11B-I). Stimuli-induced apical and basolateral secretion of the pro-inflammatory neutrophil chemokine IL8 was reduced by the postbiotic, with no difference seen between 10% and 25% doses (Fig. 11B & C). Additionally, stimuli-induced apical and basolateral secretion of the chemokines CCL2 (Fig. 11D & E) andCCL7 (Fig. 11F & G), and the pro-inflammatory cytokine TNFa (Fig. 11H & I) were completely blocked by both doses of postbiotic. Finally, stimuli-indued basolateral secretion of the chemokine CXCL5 was inhibited by the postbiotic, with a stronger inhibitory effect observed with the 25% dose (Fig. 11J).

A second organoid co-culture experiment was conducted using *B. infantis* derived HMO postbiotic (Fig. 12). TEERs increased across all conditions following the 3 hour incubation (Fig. 12A). The 'Postbiotic (25%) and Stimuli' condition produced the highest TEER, and this was significantly different from both 'Control' and 'Stimuli'. The 'Postbiotic (25%)' condition was also significantly different from 'Control'. Taken together, these results suggest that the postbiotic did exert an effect upon monolayer as the *C. perfringens* postbiotic did. However, in contrast to the *C. perfringens* postbiotic, there was no significant difference between the postbiotic in isolation and 'Stimuli', meaning the activity of this postbiotic may be weaker than that of *C. perfringens*. The *B. infantis* postbiotic also failed to inhibit stimuli-induced apical secretion of IL8, CCL7 and TNFa from the monolayers (Fig. 12B, F & H), as well as basolateral secretion of CXCL5 (Fig. 12J). It did however reduce basolateral secretions of IL8, CCL7 and TNFa (Fig. 12 B, G & I), as well as apical and basolateral secretion of CCL2 (Fig. 12 D & E). For apical and basolateral CCL2 and basolateral CCL7 the 10% dose had no significant inhibitory effect.

TEERs are widely accepted as reliable quantitative indicators of the integrity of cellular barriers and are regularly used to assess barrier function within *in vitro* gastrointestinal (GI) tract models (Srinivasan et al., J Lab Autom. 2015 April ; 20(2): 107–126). Decreases in TEER have been directly associated with decreases in expression of tight junction proteins, increases in barrier permeability and increased secretion of inflammatory cytokines in gut models (Ghosh et al., Cell Mol Gastroenterol Hepatol 2021;11:1463–1482). The inverse is also the case, that increased TEERs are associated with tightened cell junctions. Intestinal epithelial integrity is particularly critical in preterm neonates, whose GI tracts are physiologically immature and deficient in their barrier function and so are known to be "leaky" (Ma et al., mBio 2022, 13:e01299-22). Intestinal bacteria are therefore more freely able to translocate across the gut epithelium of these infants, causing infection and intestinal injury, which may in turn lead to NEC or sepsis. Promoting epithelial barrier integrity and maturation may therefore reduce risk of severe disease in preterm infants. In this context, the *C. perfringens* postbiotic induced an increase in TEER and therefore monolayer integrity, meaning it would be suitable for such a therapeutic application. Additionally, it outperformed the *B. infantis* postbiotic, which had a weaker impact on TEER that could not be fully distinguished from negative and stimuli-only controls.

These data also show the ability of both postbiotics to inhibit inflammatory cytokine secretion. Within an *in vivo* model, such inhibitory effects would logically result in reduced recruitment and activation of immune cells to sites in the gut and therefore reduced inflammation. This could aid in the prevention of inflammation associated diseases, such as

NEC, or help ameliorate the symptoms of such diseases. The *C. perfringens* postbiotic also outperformed the *B. infantis* postbiotic in this activity, by inhibiting both apical and basolateral secretion of four of the cytokines, compared to only one for the *B. infantis* postbiotic. But the *B. infantis* postbiotic did match the *C. perfringens* postbiotic in terms of inhibiting basolateral secretion of all five cytokines. It is also notable that the 10% dose of the *C. perfringens* postbiotic showed stronger inhibition of cytokine secretion than the 10% dose of the *B. infantis* postbiotic. Taken together, the cytokine and TEER data point to a *Clostridium* derived postbiotic having more potent beneficial biological activity than one derived from *Bifidobacterium*.

Example VII

Experimental design

To identify which SCFAs *Clostridium spp.* can produce when metabolising HMOs and to compare and contrast the repertoire and level of HMO-derived SCFA production of *Clostridium* with those of *Bifidobacterium*.

Methods

SCFA profiling of *Clostridium* and *Bifidobacterium* culture supernatants

For isolates found to grow on HMOs following the above growth curve protocol, supernatants from wells where bacterial growth was observed were harvested, centrifuged at 5000 G for 5 mins at 4°C to remove any bacteria and the supernatants collected. Quantitative measurement of SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and hexanoic acid) in the collected samples was then performed by Creative Proteomics, USA, using a GC-MS method. Samples were diluted in water containing labelled internal standards for each chain length (C2- C6). The free short chain fatty acids were derivatized using methyl chloroformate in 1- propanol yielding propyl esters before subsequent liquid-liquid extraction into hexane and analysis on a SLB-5ms (30 × 0.25 mm × 1.0µm) column and detection using GC-EI-MS in SIMmode. The analytes were quantified using 8-point calibration curves. Pairwise comparisons between *Bifidobacterium* and *Clostridium* for mean relative SCFAs levels were performed using an unpaired Student's T test in R, with $p < 0.05$ set at the threshold for statistical significance.

Results

Of the seven SCFAs quantified in supernatants, *Clostridium* produced significantly greater quantity of four of them compared to *Bifidobacterium* (propionate, butyrate, valerate and hexanoate) (Fig. 13). *Bifidobacterium* failed to produce propionate, butyrate or valerate. *Bifidobacterium* produced mainly acetate in large quantities, although *Clostridium* was also able to produce this SCFA in relative high levels compared to the other SCFAs. Both genera produced only minimal quantities of the branched SCFAs isobutyrate and isovalerate. These branched SCFAs are considered to primarily be products of amino acid catabolism (Oliphant & Allen-Vercoe, Microbiome 2019, 7:1:91), rather than carbohydrate catabolism as with the other SCFAs, so their lower abundance in supernatants from cultures given a plentiful supply of HMOs is explicable. The wider repertoire and lower uniformity of SCFA production within the *Clostridium* is further evidenced by the wide datapoint spread and confidence region of the genus during unsupervised ordination, compared to the tight grouping of *Bifidobacterium* samples (Fig. 13H). Furthermore, *Clostridium* and *Bifidobacterium* predominantly cluster separately from one another during both ordination and dendrogram analyses, highlighting the distinctiveness of the SCFA production profiles of the two genera (Fig. 13H & I).

Production of SCFAs is important to human health, as these compounds have a range of beneficial effects in the host, such as suppression of inflammatory cytokine secretion, promotion of intestinal barrier function and promotion of colonocyte metabolic function (Cifuentes et al., Current Research in Microbial Sciences 2024, 6:100219). They impact immune, nervous, and metabolic systems within the host. More specifically for neonates, SCFAs are associated with the health benefits to newborns of breastfeeding, as *Bifidobacterium spp.* are known to primarily metabolise HMOs into SCFAs (Stewart, Nat. Microbiol. 2021, 6 (11), 1335–1336). These data demonstrate that *Clostridium* can metabolise HMOs to produce greater quantities and a wider range of SCFAs than *Bifidobacterium* and that the genus contains greater heterogeneity in SCFA production than *Bifidobacterium*. Therefore, *Clostridium* could compensate for any absence of *Bifidobacterium* SCFA production, as they also produce high quantities of acetate, but the inverse is not true. Additionally, the heterogeneity amongst *Clostridium* raises the notion that it is important to identify the "right" *Clostridium* pro- or postbiotic, to ensure an optimal SCFA profile. Finally, these data show that the *Clostridium* postbiotics described herein will contain high quantities of various SCFAs, which may be mediating some of the beneficial activities outlined above.

Example VIII

Experimental design

Use untargeted metabolomics, performed by Metabolon Inc., to identify and compare the metabolites present within *Clostridium* and *Bifidobacterium* derived postbiotics. To identify the metabolites present within *Clostridium* postbiotics that may be responsible for the beneficial activities described above.

Methods

Generation of postbiotics

Postbiotics were generated as described above. Biological triplicates of each were produced and sent for metabolomics analysis.

Sample Preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into multiple fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, while the remaining fractions were reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.
QA/QC: Several types of controls were analysed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analysed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall

process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution (PMID: 32445384). The dried sample extract were then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analysed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds (PosEarly). In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analysed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds (PosLate). In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analysed using basic negative ion optimized conditions using a separate dedicated C18 column (Neg). The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analysed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8 (HILIC). The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Bioinformatics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information

interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

LIMS

The purpose of the Metabolon LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

Data Extraction and Compound Identification

Raw data was extracted, peak-identified and QC processed using a combination of Metabolon developed software services (applications). Each of these services perform a specific task independently, and they communicate/coordinate with each other using industry-standard protocols. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and fragmentation data on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between molecules based on one of these factors, the use of all three data points is utilized to distinguish and differentiate biochemicals. More than 5,400 commercially available purified or in-house synthesized standard compounds have been acquired and analysed on all platforms for determination of their analytical characteristics. An additional 7000 mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis. Metabolon continuously adds

biologically-relevant compounds to its chemical library to further enhance its level of Tier 1 metabolite identifications.

Compound Quality Control

A variety of curation procedures were carried out to ensure that a high-quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove or correct those representing system artifacts, mis-assignments, mis-integration and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification and integration among the various samples.

Metabolite Quantification and Data Normalization

Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (termed the "block correction"). For studies that did not require more than one day of analysis, no normalization is necessary, other than for purposes of data visualization. In certain instances, biochemical data may have been normalized to an additional factor (e.g., cell counts, total protein as determined by Bradford assay, osmolality, etc.) to account for differences in metabolite levels due to differences in the amount of material present in each sample.

Results

During unsupervised ordination and dendrogram analyses of the metabolomics data, samples predominantly clustered based on genus (Fig. 14). During ordination specifically (Fig. 14A), the *Bifidobacterium* postbiotics formed a tighter cluster with a smaller 95% confidence region compared to the *Clostridium* isolates. These outcomes mirror those seen in the SCFA analysis (Fig. 13H & I) and indicate that the two genera produced largely distinct metabolite profiles in their postbiotics and that there was much greater heterogeneity in metabolite production by *Clostridium*.

Within the dataset, it was found that multiple *Clostridium* postbiotics, specifically those generated from *C. perfringens* and *C. tertium*, contained quantities of indoles that were absent from *Bifidobacterium* postbiotics (Fig. 15), specifically indole-3-acetaldehyde, indole-3-carbaldehyde, tryptamine, 3-indoleglyoxylic acid and tryptophan. Postbiotics from *C. butyricum* (which cannot utilise HMOs) also contained two of these compounds (tryptophan and indole-3-carbaldehyde) that were statistically different from the blank medium. Additionally, some postbiotics from the same three species also contained higher quantities of oxindolylalanine, however the inconsistency of these quantities between samples from the same species meant these levels were not found to be statistically significant (Fig. 15G). All four sets of *Bifidobacterium* postbiotics were shown to contain raised levels of indolelactate (Fig. 15F), with *C. tertium* and *C. butyricum* postbiotics containing similar quantities. Indolelactate was also present in *C. perfringens* postbiotics, but to a lower level.

It was further found that *Clostridium* postbiotics contained raised quantities of polyamines, diamines and their precursors ornithine and citrulline (Fig. 16). The polyamines agmatine and spermidine were produced within *C. perfringens* postbiotics, with *C. tertium*, *C. butyricum*, *C. paraputrificum* and *C. baratii* also having produced spermidine. *Bifidobacterium* postbiotics lacked these compounds. The diamine putrescine was produced only by *C. tertium* and *C. paraputrificum*, while another diamine cadaverine was found in all glucose postbiotics of both genera (the grouping of glucose and HMO postbiotics in the plots means cadaverine levels did not show statistical differences from the blank medium). The non-proteinogenic amino acids ornithine and citrulline had raised levels in *B. infantis* postbiotics, but not in any other *Bifidobacterium* sample. Similar levels of ornithine were also produced by *C. perfringens* and *C. paraputrificum* and slightly lower levels, but still raised vs blank medium and other species, by *C. tertium*. *C. perfringens* postbiotics also on average contained similar levels of citrulline to *B. infatis* postbiotics. The ornithine and agmatine precursor arginine was found to be far lower on average in *C. perfringens* postbiotics.

Additional metabolites of interest were also found to be present at relatively high levels within *Clostridium* postbiotics (Fig. 17). Significantly higher levels of either butyrate or isobutyrate (Metabolon's chromatography methodology is unable to distinguish between structural isomers) were detected in all *Clostridium* postbiotics, with no statistical difference between the *Bifidobacterium* and blank medium (Fig. 17A). *Clostridium* and *Bifidobacterium* postbiotics did however all share increased levels of phenyllactate, with the four *Bifidobacterium spp.*, *C. tertium* and *C. butyricum* producing similarly high amounts, followed by *C. perfringens* and *C. paraputrificum* (Fig. 17B). *C. baratii* produced the least phenyllactate and was not statistically different from the blank medium. Finally, *C.*

perfringens postbiotics were shown to contain significantly raised levels of histamine compared to the blank and all other isolates, with no other species producing this metabolite significantly above the level measured in the blank medium (Fig. 17C).

Further identified metabolites of interest were 3-hydroxybutyrate and trimethylamine N-oxide, both of which were raised in *Clostridium* postbiotics (Fig. 18) while being at very low abundance in all *Bifidobacterium* postbiotics. As with many of the metabolites above, *C. perfringens* and *C. tertium* postbiotics contained the greatest quantities of these two compounds, with *C. paraputrificum* and *C. butyricum* also containing raised levels of 3-hydroxybutyrate.

Indole-containing compounds are known to be produced from tryptophan catabolism by gut bacteria and they and their derivatives exert profound and beneficial effects on the host, such as promoting intestinal barrier function and inhibiting inflammation (Li et al., Front. Pharmacol. 2021, 12:769501). *Bifidobacterium spp.* are known to be producers of these compounds and it is thought they may help mediate the beneficial health effects associated with the genus (Sakurai et al., Microorganisms 2019, 7, 340). The finding in the metabolomics data that multiple of the *Clostridium* species produced these compounds within their postbiotics is therefore noteworthy. Not only were *Clostridium* able to produce the same indole compound as the *Bifidobacterium*, namely indolelactate, but they were able to produce a wider range of these beneficial compounds. Indole-3-carbaldehyde is known to stimulate production of the cytokine IL22 in the gut, which promotes healthy host-microbe homeostasis in the gut mucosa (Zhang & Davies, Genome Med. 2016; 8: 46). Indole-3-acetaldehyde is considered to be chemically unstable and detectable levels of it are therefore thought to transient, with the compound usually converted into indole-3-acetate (Nutaratat et al., Arch Microbiol 2016, 198:429–437). If this conversion takes place, the resulting indole-3-acetate is known to be able to suppress inflammatory responses in immune cells (Li et al., Front. Pharmacol. 2021, 12:769501). Tryptamine can activate serotonin receptors in the human gut, triggering the opening of ion channels and increasing electrolyte secretion, which promotes intestinal motility (Bhattarai et al., Cell Host & Microbe 2018, 23, 775–785). Specific activities related to gut health do not appear to have been attributed to 3-indoleglyoxylic acid yet, but derivatives of this compound are known to have antiemetic activities (Huang et al., Abstracts of Papers of the American Chemical Society 1993, Vol.206). Furthermore, the increased levels of tryptophan in multiple *Clostridium* postbiotics raises the prospect that some of these species (*C. perfringens*, *C. tertium* and *C. butyricum*) are able to synthesise this amino acid. Other gut species would be able to metabolically cross-feed on tryptophan that is either supplemented in the gut via a postbiotic

or being actively produced by a live probiotic strain. Such cross-feeding could accordingly increase the levels of beneficial indole compounds and further promote gut health.

Polyamines, including spermidine and agmatine, are produced from lysine via cadaverine, and from arginine, ornithine and citrulline via putrescine (Sagar et al., Med. Sci. 2021, 9, 44). They are known to have numerous beneficial activities within the gut, such as increasing expression of tight junction proteins and reducing inflammatory immune responses (Rooks & Garrett, Nat Rev Immunol 2016, 16, 341–352; Pruss et al., Nat Metab 2022, 4, 19–28). Synthesis of polyamines has been identified as a vital gut microbiome function that is necessary for healthy postnatal development of the GI tract. Furthermore, supplementation of polyamine precursors, namely arginine and citrulline, in tandem with probiotic strains has been shown to boost polyamine levels, decrease secretion of pro-inflammatory TNFa and protect against adhesion of enterohemorrhagic and enteroinvasive *E. coli* (Rooks & Garrett, Nat Rev Immunol 2016, 16, 341–352; Ho et al., Journal of Functional Foods 2020, 64 103571). *Clostridium* postbiotics contained a wider range of this group of compounds, suggesting the biosynthetic pathways responsible for them were more highly productive in this genus compared to *Bifidobacterium*. The presence of citrulline and ornithine in *B. infantis* postbiotic samples demonstrates these pathways were active in this species, but the absence of raised spermidine, putrescine and agmatine suggests *B. infantis* is at least not as efficient a producer of polyamines as *C. perfringens, C. tertium*, *C. butyricum* and *C. paraputrificum*. The lower levels of arginine in *C. perfringens* postbiotics further indicates strains of this species to be potent metabolisers of this amino acid and it appears this metabolism is channelled into ornithine and polyamine biosynthesis.

The higher levels of the SCFA butyrate/isobutyrate observed in *Clostridium* postbiotics mirrors the SCFA profiling results shown in Fig. 13, so confirms that members of this genus are prolific producers of SCFAs. The potential benefits to the host of high SCFA production have been discussed above. Phenyllactate is a well-known metabolite of lactic acid bacteria, which are often utilised as probiotic strains, and has been found to have broad spectrum antimicrobial activity against both Gram-negative and Gram-positive species (Ning et al., Food Chemistry 2017, 228, 533–540; Sun et al., Biochemical and Biophysical Research Communications 2019, 519, 351e357). Production of this metabolite is yet another feature that *C. perfringens*, *C. tertium*, *C. butyricum* and *C. paraputrificum* share with *Bifidobacterium*. The presence of phenyllactate could explain the inhibition of pathobiont growth observed with *Clostridium* derived postbiotics in Example III above.

Histamine is known to be produced by *Lactobacillus* strains used as live probiotics and has been shown to suppress cytokine secretion and wider intestinal inflammation, through binding to the H2R histamine receptor (Goa et al., mBio 2015, 6(6):e01358-15). However, the immunological effects of histamine do depend on which of the four histamine receptors is triggered and all of these are expressed within the human gut (Deiteren et al., British Journal of Pharmacology 2015, 172 1165–1178; Barcik et al., Current Opinion in Immunology 2017, 48:108–113). It is therefore acknowledged that while histamine-producing probiotics possess anti-inflammatory effects, greater understanding of these effects is required. In these data, *C. perfringens* postbiotics contained higher histamine than all others. A study in fish previously found *C. perfringens* produced histamine below the threshold for toxicity (Bjornsdottir-Butler et al., J. Food Prot. 2013, 76:7). Therefore, the histamine within the *C. perfringens* postbiotic may be optimal for exerting a beneficial anti-inflammatory effect.

Both 3-hydroxybutyrate and trimethylamine N-oxide are known neurotransmitters that have been shown to be enriched within the intestines of neonatal mice (Sanidad et al., Sci. Immunol. 2024, 9, eadj4775). While the developmental and immune impacts of microbially-derived neurotransmitters are yet to be fully explained, the gut microbiome has been found to have a potential role in regulating neuroinflammation and brain activity and thus could impact neurodevelopment via signalling through metabolites such as these (Bostick et al., Current Opinion in Immunology 2022, 76; Needham et al., Nature 2022, 602, 647–653).

The combined beneficial activities of the metabolites described above could account for the anti-inflammatory, barrier function promoting and pathobiont inhibitory effects observed from *C. perfringens* and *C. tertium* postbiotics in Examples III and VI. Especially since *C. perfringens* and *C. tertium* postbiotics typically contained some of the highest quantities of these specified metabolites. Furthermore, as with the SCFAs in Example VII, these species, alongside *C. butyricum* and *C. paraputrificum*, often outperformed or matched *Bifidobacterium spp.* in terms of the ranges and quantities of these metabolites that they produced within their postbiotics.

Taking a global view of the metabolomics data, it is clear from the dendrogram (Fig. 14B) that samples cluster primarily by strain and as such the differences between HMO and glucose postbiotics were minimal. And as with the SCFA data, the *Clostridium* were far more heterogeneous than the *Bifidobacterium*. This raises again a point from Example VII and the SCFA data that different *Clostridium* could be used as a pro- or postbiotic to generate an optimal and tailored metabolite composition.

The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings), may be replaced by alternative features serving the same, equivalent, or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

Claims

1. A probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* further comprising at least one prebiotic; wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

2. The probiotic composition according to claim 1, wherein the composition comprises at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

3. The probiotic composition according to claim 1, wherein the composition comprises at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

4. The probiotic composition according to any preceding claim, wherein the composition comprises at least 0.5 x 10^9 CFU *Clostridium* species per dose.

5. The probiotic composition according to any preceding claim, wherein the MMO is a human milk oligosaccharide (HMO) a precursor and/or a derivative thereof.

6. The probiotic composition according to claim 5, wherein the HMO is selected from the group consisting of: lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), disialyllacto-N-tetraose (DSLNT), lacto-N-fucopentaose I (LNFPI), 6'-sialyllactose (6SL) and 2-fucosyllactose (2FL); or a combination thereof; optionally wherein the HMO is LNnT and/or 6SL.

7. A postbiotic composition for improving the gut health of a subject, the composition comprising:
(a) at least one inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or
(b) metabolites from at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

8. The postbiotic composition of claim 7, wherein the composition comprises:

(a) at least two inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or
(b) metabolites from at least two *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

9. The postbiotic composition of claim 7, wherein the composition comprises:
(a) at least three inanimate *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum*; and/or
(b) metabolites from at least three *Clostridium* species selected from the group consisting of: *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and *Clostridium paraputrificum*.

10. The probiotic composition or postbiotic composition according to any one of the preceding claims, wherein
a) the *Clostridium perfringens* species is selected from the group consisting of: *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); and/or
b) the *Clostridium baratti* species is *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908); and/or
c) the *Clostridium tertium* species is selected from the group consisting of: *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905) and *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911); and/or
d) the *Clostridium paraputrificum* species is selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902) and *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910).

11. The probiotic composition or postbiotic composition according to any one of the preceding claims, wherein the composition is in the form of a liquid medium, gel medium, powder, dissolvable medium, chewable medium, or any other ingestible medium.

12. The probiotic composition or postbiotic composition according to claim 11, wherein the composition is a milk product.

13. The probiotic composition or postbiotic composition according to claim 12, wherein the composition is an infant formula, a starter infant formula, a follow-on or follow-up infant formula, a baby food, or an infant cereal composition.

14. The probiotic composition or postbiotic composition according to any one of claims 11 to 13, wherein the composition is a powder, a liquid or a solid.

15. A probiotic composition or postbiotic composition of any preceding claim for use in improving the gut microbiome and/or the gut health of a subject.

16. The probiotic composition for use, or postbiotic composition for use, according to claim 15, wherein the subject is an infant.

17. The probiotic composition for use, or postbiotic composition for use, according to claim 16, wherein the infant is a neonate, a preterm infant, an infant small for gestational age, or an infant born by C-section.

18. The probiotic composition for use, or postbiotic composition for use, according to claim 16 or claim 17, wherein the infant is hospitalised and/or is undergoing antibiotic treatment.

19. The probiotic composition for use, or postbiotic composition for use, according to any one of claims 15 to 18, wherein the composition reduces pathogen overgrowth in the gut, promotes gut development, promotes gut maturation, promotes immunity, decreases gut inflammation, and/or promotes enteral feeding tolerance in the subject.

20. A probiotic composition or postbiotic composition of any one of claims 1 to 14 for use in preventing or treating a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof, in a subject.

21. A probiotic composition for use in preventing or treating a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof, in a subject;

wherein the probiotic composition comprises at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* and

the subject consumes at least one prebiotic prior, after, or during administration of the probiotic composition, wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

22. A method of treating or preventing a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof in a subject, comprising administering a therapeutically effective amount of the probiotic composition or the postbiotic composition of any one of claims 1 to 14 to said subject.

23. A method of treating or preventing a gastrointestinal disorder, an allergy, nappy rash, colic, or a symptom thereof in a subject, comprising administering a therapeutically effective amount of a probiotic composition prior, after, or during administration of a prebiotic composition,

wherein the probiotic composition comprises at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* and

wherein the prebiotic is a mammalian milk oligosaccharide (MMO), a precursor and/or a derivative thereof.

24. The probiotic composition for use, or postbiotic composition for use, according to claim 20 or 21, or the method according to claim 22 or 23, wherein the gastrointestinal disorder affects the integrity of the intestinal lining.

25. The probiotic composition for use, or postbiotic composition for use according to claim 20, 21 or 24, or the method according to claim 22 to 24, wherein the gastrointestinal disorder is selected from the group consisting of: intestinal inflammation, irritable bowel syndrome, colitis, necrotising enterocolitis, late onset sepsis, focal intestinal perforation, and inflammatory bowel disease.

26. The probiotic composition for use, or postbiotic composition for use, according claim 25, or the method according to claim 25, wherein the gastrointestinal disorder is necrotising enterocolitis.

27. The probiotic composition for use, or postbiotic composition for use, according to any one of claims 20, 21, or 24 to 26, or the method according to any one of claims 22 to 26, wherein the subject is an infant.

28. The probiotic composition for use, or postbiotic composition for use, or the method, according to claim 27, wherein the infant is a neonate, a preterm infant, an infant small for gestational age, or an infant born by C-section.

29. The probiotic composition for use, or postbiotic for use, or the method, according to claim 27 or claim 28, wherein the infant is hospitalised and/or is undergoing antibiotic treatment.

30. A probiotic composition for improving the gut microbiome and/or gut health of a subject, the composition comprising at least one *Clostridium* species selected from the group consisting of: *Clostridium perfringens*, *Clostridium baratii, Clostridium tertium* and *Clostridium paraputrificum;* wherein

a) the *Clostridium perfringens* species is selected from the group consisting of: *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); and/or

b) the *Clostridium baratti* species is *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908); and/or

c) the *Clostridium tertium* species is selected from the group consisting of: *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905) and *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911); and/or

d) the *Clostridium paraputrificum* species is selected from the group consisting of: *Clostridium paraputrificum* strain JC53 (deposited under NCTC accession number 24022902) and *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910).

31. Use of *Clostridium perfringens, Clostridium baratii*, *Clostridium tertium* and/or *Clostridium paraputrificum* as a probiotic or postbiotic.

32. *Clostridium perfringens*, *Clostridium baratii*, *Clostridium tertium* and/or *Clostridium paraputrificum,* or a *Clostridium* postbiotic thereof, for use in improving the gut microbiome and/or gut health in a subject.

33. A bacterial strain selected from the group consisting of:
Clostridium paraputrificum strain JC53 (deposited under NCTC accession number 24022902), *Clostridium perfringens* strain AM1 (deposited under NCTC accession number 24022903), *Clostridium perfringens* strain JC26 (deposited under NCTC accession number 24022904), *Clostridium tertium* strain JC25 (deposited under NCTC accession number 24022905), *Clostridium perfringens* strain AM248 (deposited under NCTC accession number 24022906), *Clostridium perfringens* strain JC36 (deposited under NCTC accession number 24022907), *Clostridium baratti* strain AM37 (deposited under NCTC accession number 24022908), *Clostridium butyricum* strain JC31 (deposited under NCTC accession number 24022909), *Clostridium paraputrificum* strain JC66 (deposited under NCTC accession number 24022910), *Clostridium tertium* strain TB43 (deposited under NCTC accession number 24022911), *Clostridium perfringens* strain JC33 (deposited under NCTC accession number 24022912), *Clostridium perfringens* strain AM226 (deposited under NCTC accession number 24022913), and *Clostridium perfringens* strain JC29 (deposited under NCTC accession number 24022914); or a derivative thereof.

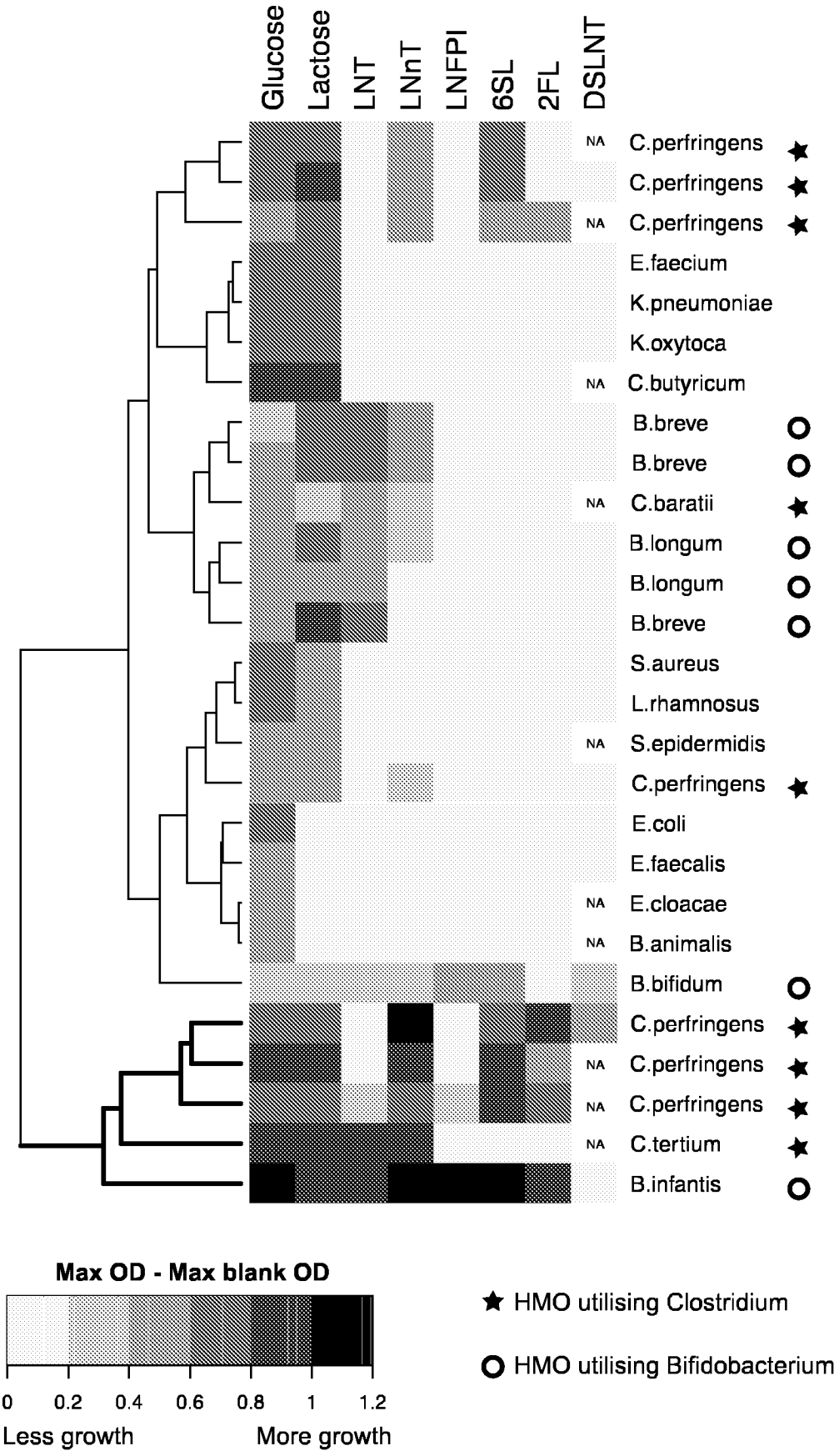

Glucose
Lactose
LNT
LNnT
LNFPI
6SL
2FL
DSLNT
NA C.perfringens
C.perfringens
NA C.perfringens
E.faecium
K.pneumoniae
K.oxytoca
NA C.butyricum
B.breve
B.breve
NA C.baratii
B.longum
B.longum
B.breve
S.aureus
L.rhamnosus
NA S.epidermidis
C.perfringens
E.coli
E.faecalis
NA E.cloacae
NA B.animalis
B.bifidum
C.perfringens
NA C.perfringens
NA C.perfringens
NA C.tertium
B.infantis
Max OD - Max blank OD
0 0.2 0.4 0.6 0.8 1 1.2
Less growth
More growth
HMO utilising Clostridium
HMO utilising Bifidobacterium

FIG. 1A

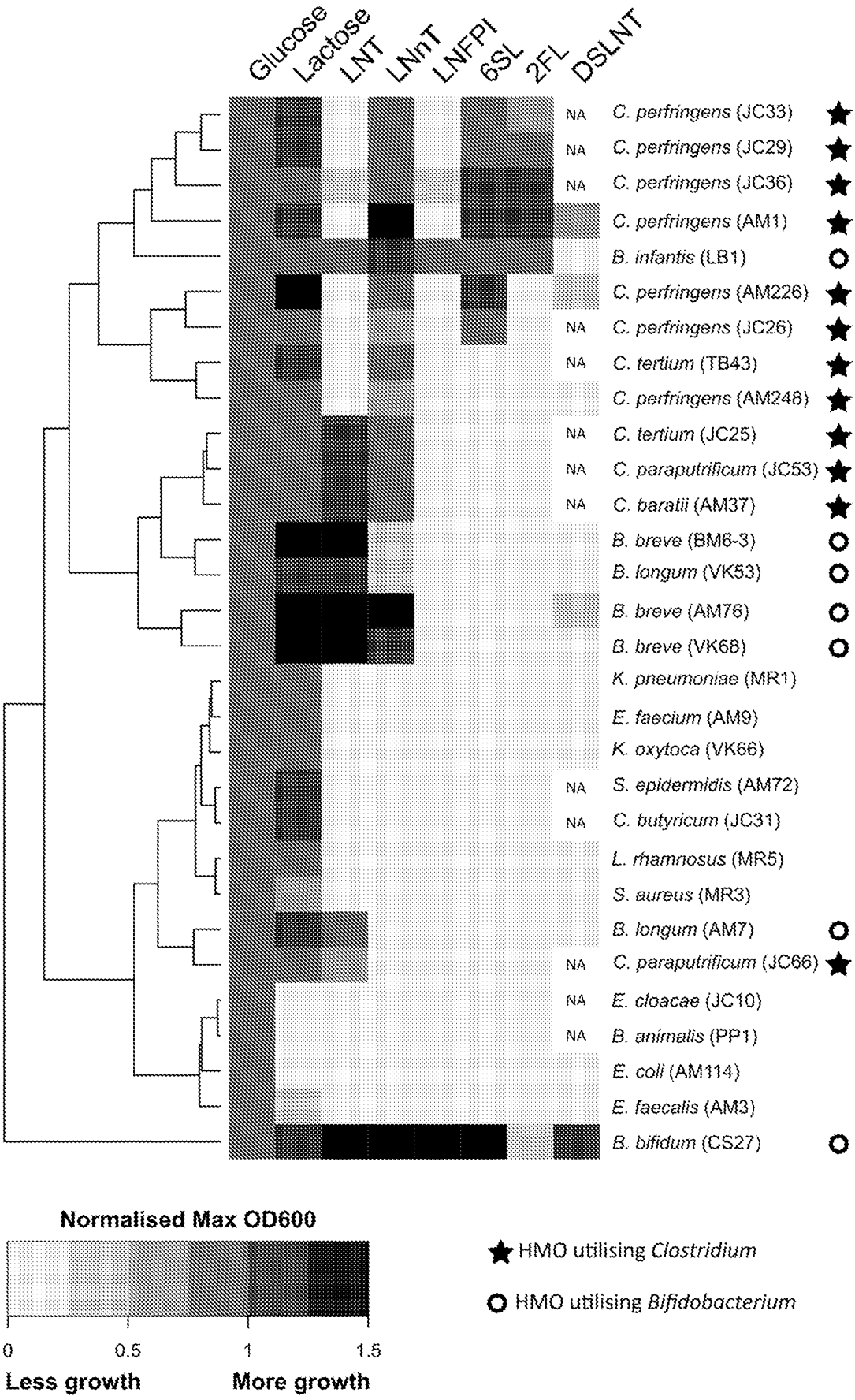

Glucose
Lactose
LNT
LNnT
LNFPI
6SL
2FL
DSLNT
NA C. perfringens (JC33)
NA C. perfringens (JC29)
NA C. perfringens (JC36)
C. perfringens (AM1)
B. infantis (LB1)
C. perfringens (AM226)
NA C. perfringens (JC26)
NA C. tertium (TB43)
C. perfringens (AM248)
NA C. tertium (JC25)
NA C. paraputrificum (JC53)
NA C. baratii (AM37)
B. breve (BM6-3)
B. longum (VK53)
B. breve (AM76)
B. breve (VK68)
K. pneumoniae (MR1)
E. faecium (AM9)
K. oxytoca (VK66)
NA S. epidermidis (AM72)
NA C. butyricum (JC31)
L. rhamnosus (MR5)
S. aureus (MR3)
B. longum (AM7)
NA C. paraputrificum (JC66)
NA E. cloacae (JC10)
NA B. animalis (PP1)
E. coli (AM114)
E. faecalis (AM3)
B. bifidum (CS27)
Normalised Max OD600
0
0.5
1
1.5
Less growth
More growth
HMO utilising Clostridium
HMO utilising Bifidobacterium

FIG. 1B

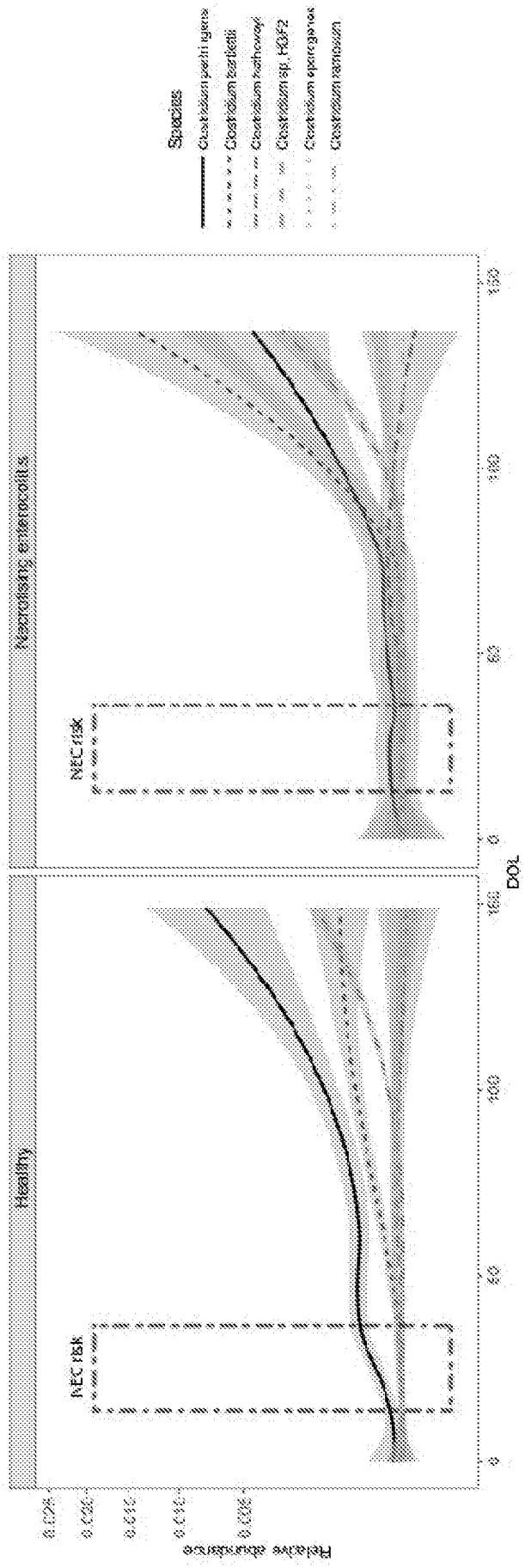

Species
Clostridium perfringens
Clostridium tertium
Clostridium butyricum
Clostridium sp. KLE2
Clostridium sporogenes
Clostridium ramosum
Necrotising enterocolitis
Healthy
NEC risk
Relative abundance
DOL

FIG. 2

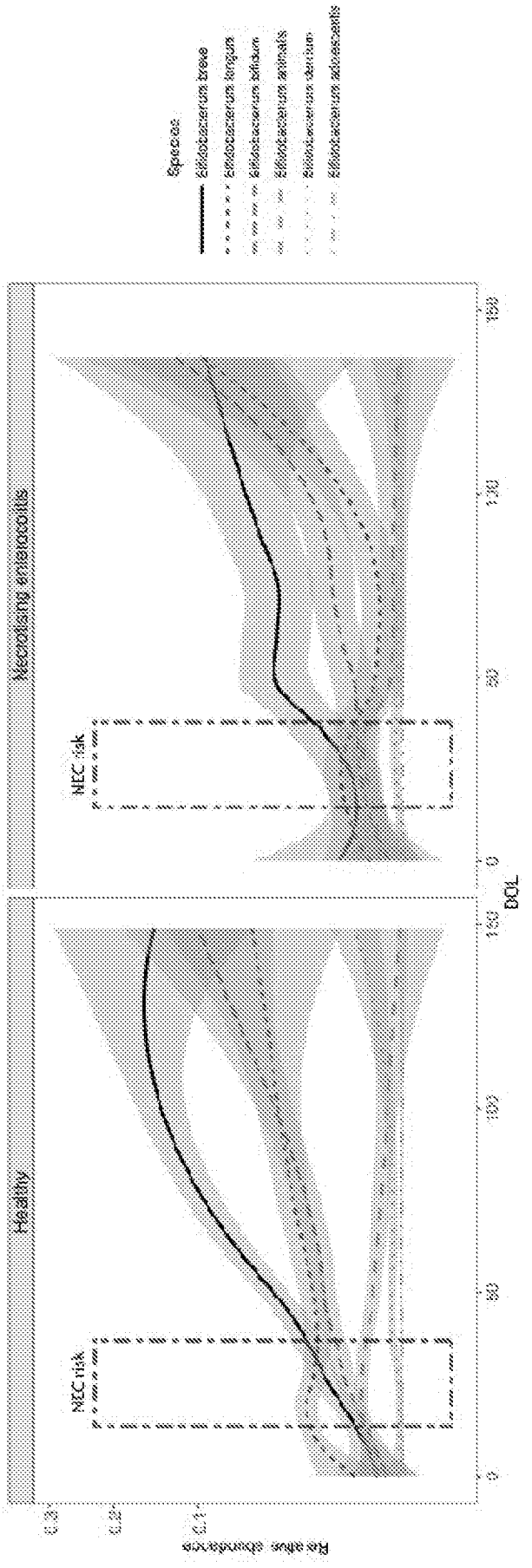

Species
Ethelcitenerum trexe
Ethelcitenerum longum
Ethelcitenerum schlum
Ethelcitenerum animalis
Ethelcitenerum dentium
Ethelcitenerum adolescentis
Healthy
Necrotising enterocolitis
MEC risk
MEC risk
0
50
100
150
0
50
100
150
DOL
0.3
0.2
0.1
Relative abundance

FIG. 3

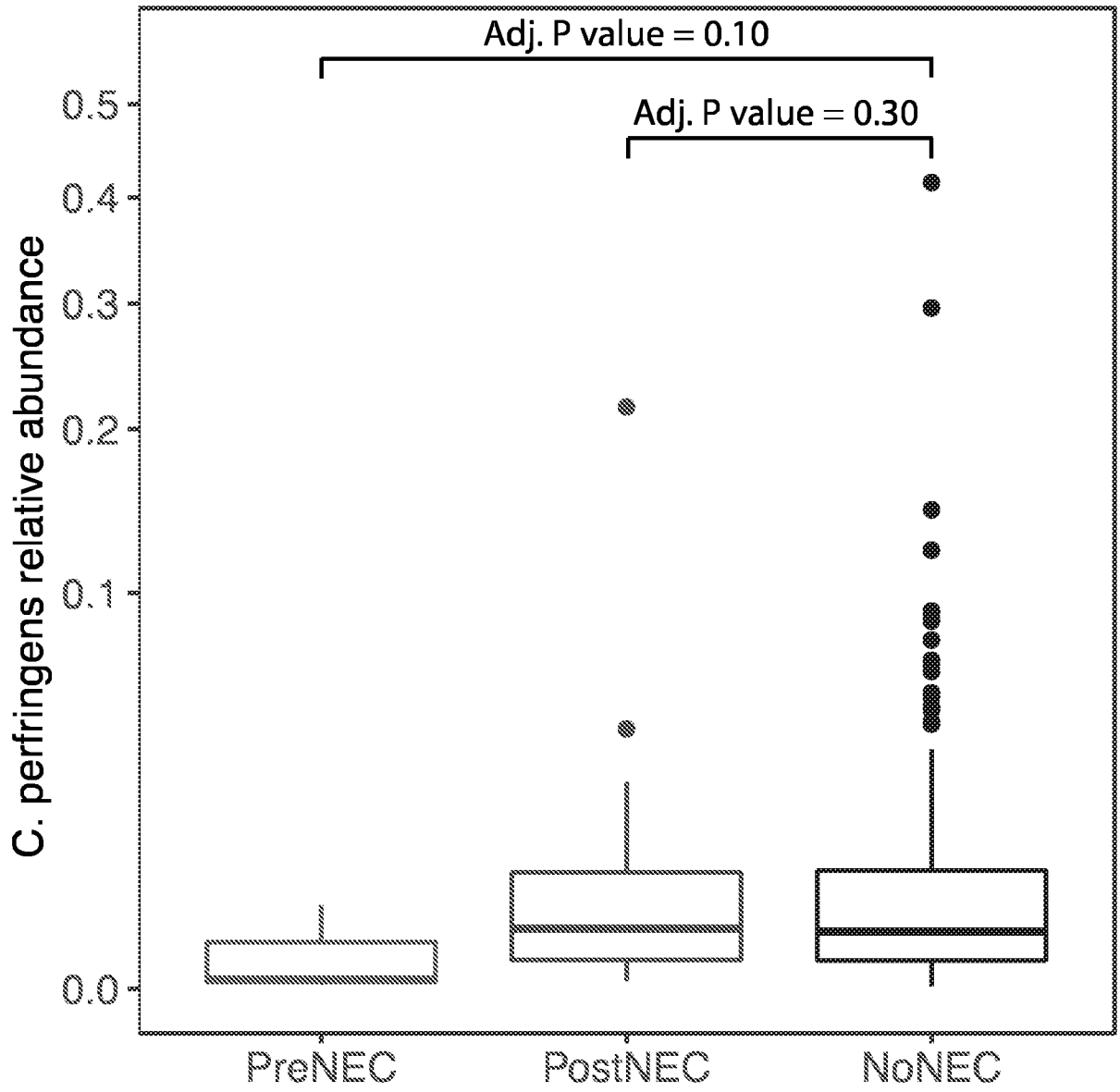

Adj. P value = 0.10
Adj. P value = 0.30
C. perfringens relative abundance
0.5
0.4
0.3
0.2
0.1
0.0
PreNEC
PostNEC
NoNEC

FIG. 4

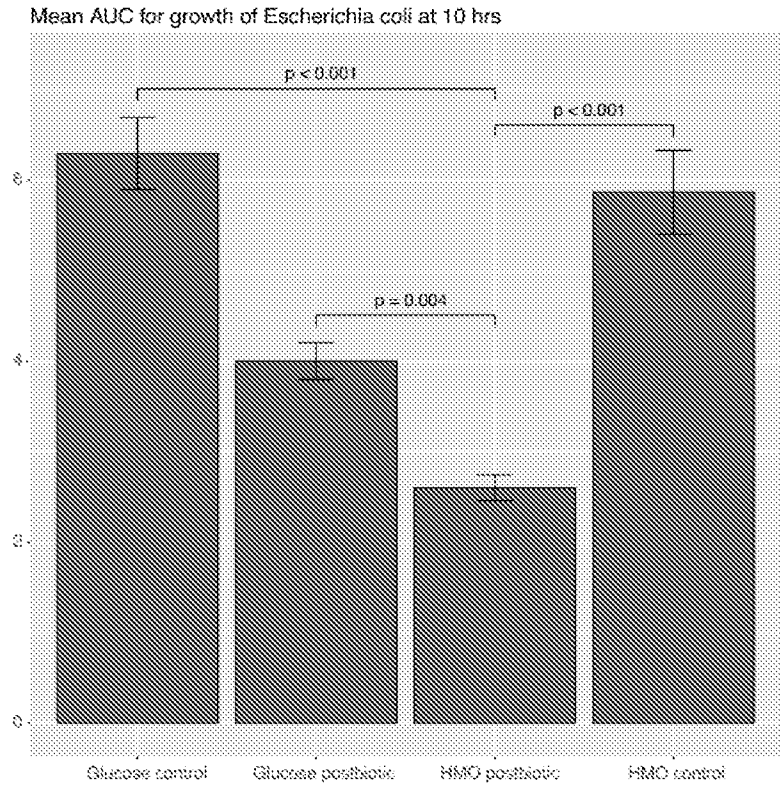

Mean AUC for growth of Escherichia coli at 10 hrs
p < 0.001
p < 0.001
p = 0.004
Glucose control
Glucose postbiotic
HMO postbiotic
HMO control

FIG. 5A

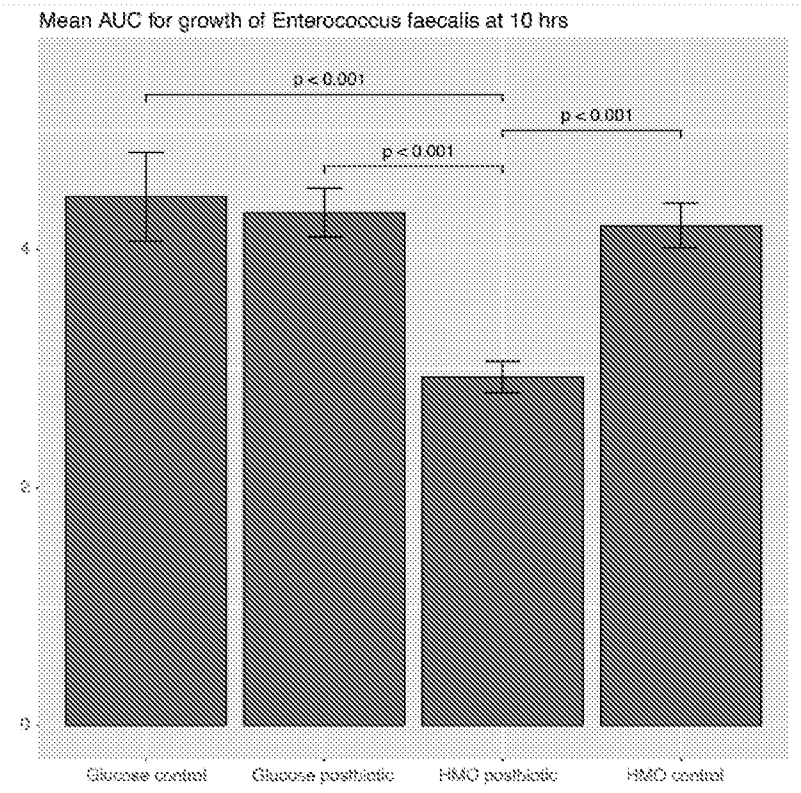

Mean AUC for growth of Enterococcus faecalis at 10 hrs
p < 0.001
p < 0.001
p < 0.001
Glucose control
Glucose postbiotic
HMO postbiotic
HMO control

FIG. 5B

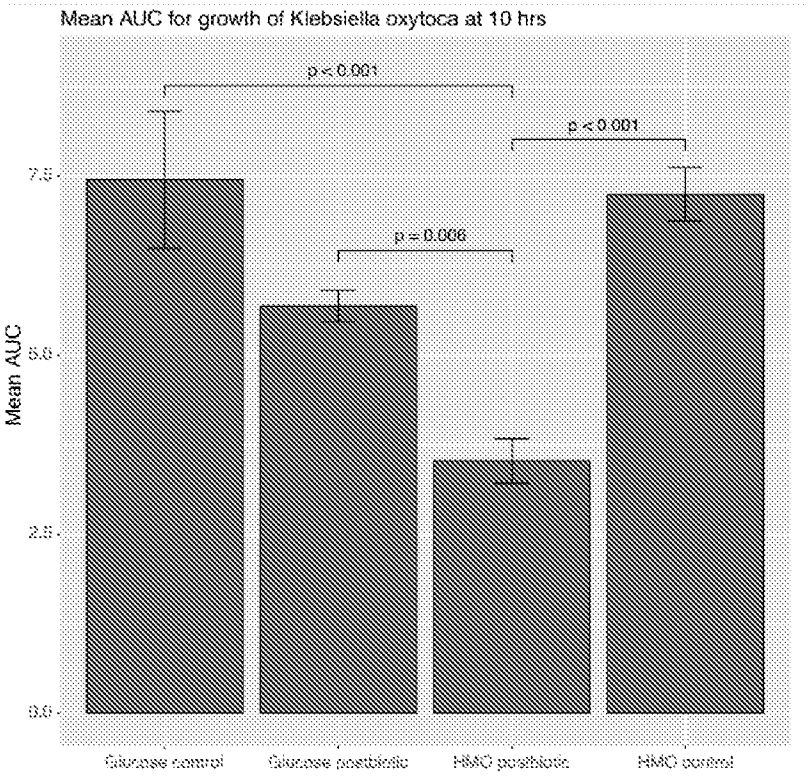

Mean AUC for growth of Klebsiella oxytoca at 10 hrs
p < 0.001
p < 0.001
p = 0.006
Mean AUC
7.5
5.0
2.5
0.0
Glucose control
Glucose postbiotic
HMO postbiotic
HMO control

FIG. 5C

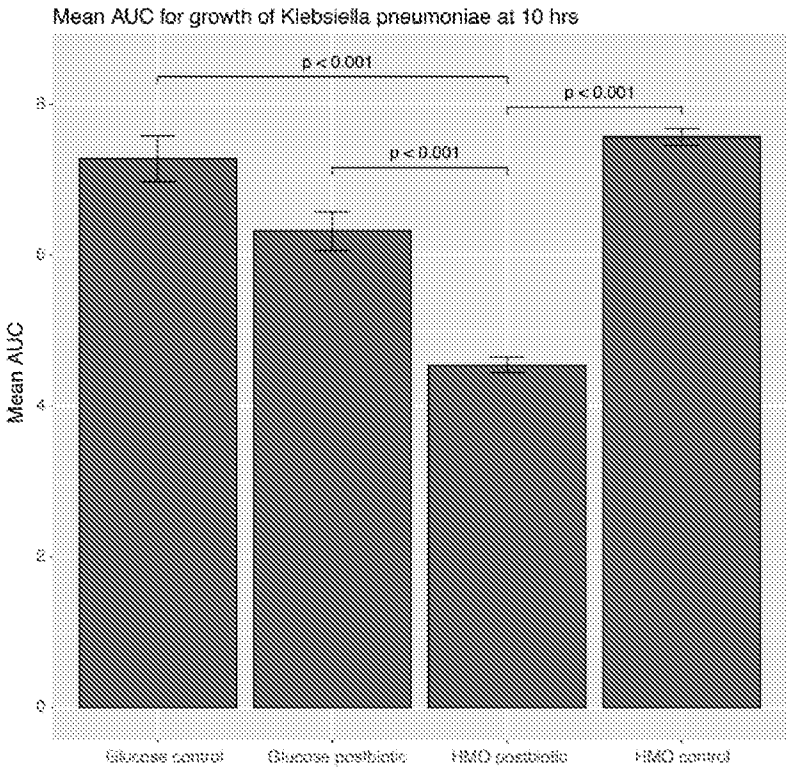

Mean AUC for growth of Klebsiella pneumoniae at 10 hrs
p < 0.001
p < 0.001
p < 0.001
Mean AUC
8
6
4
2
0
Glucose control
Glucose postbiotic
HMO postbiotic
HMO control

FIG. 5D

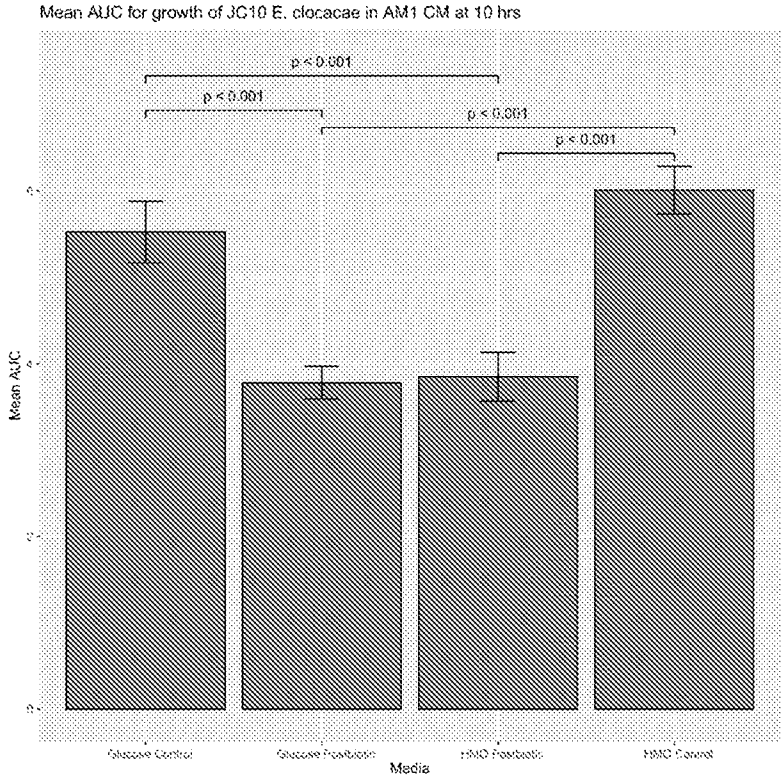

Mean AUC for growth of JC10 E. clocacae in AM1 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Glucose Control
Glucose Postbiotic
HMO Postbiotic
HMO Control
Media

FIG. 5E

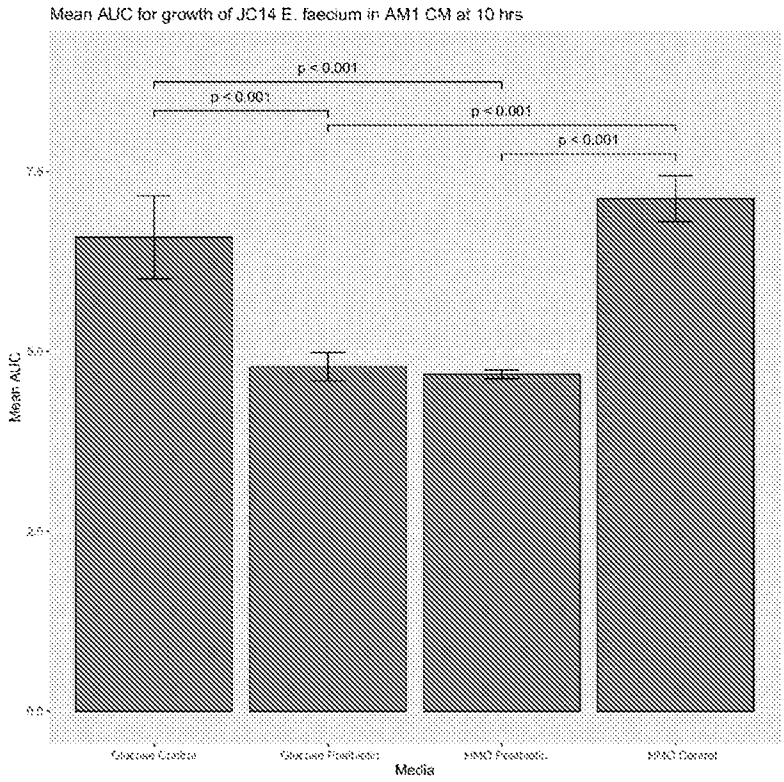

Mean AUC for growth of JC14 E. faecium in AM1 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Glucose Control
Glucose Postbiotic
HMO Postbiotic
HMO Control
Media

FIG. 5F

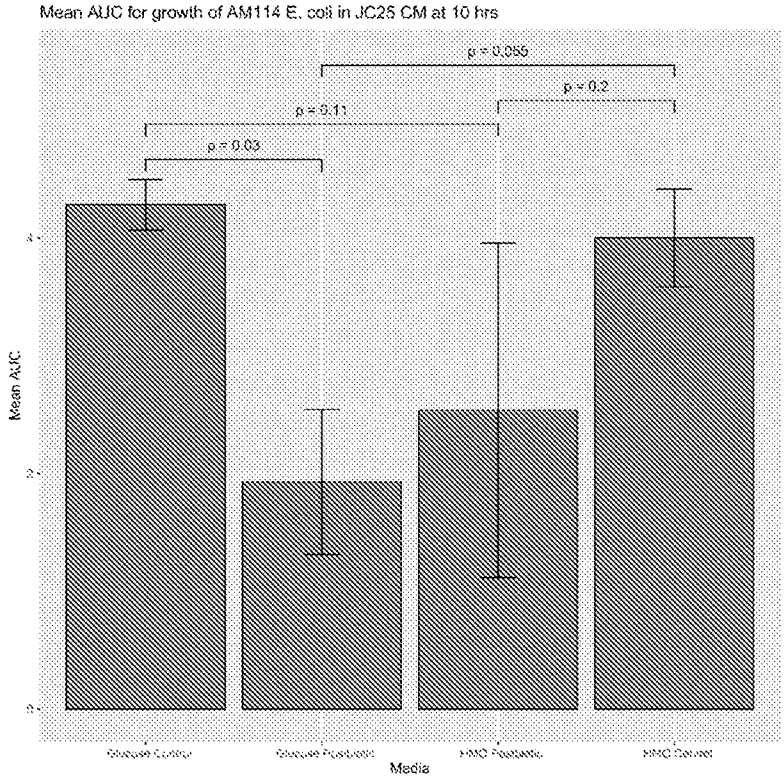

Mean AUC for growth of AM114 E. coli in JC25 CM at 10 hrs
p = 0.055
p = 0.2
p = 0.11
p = 0.03
Mean AUC
Media

FIG. 6A

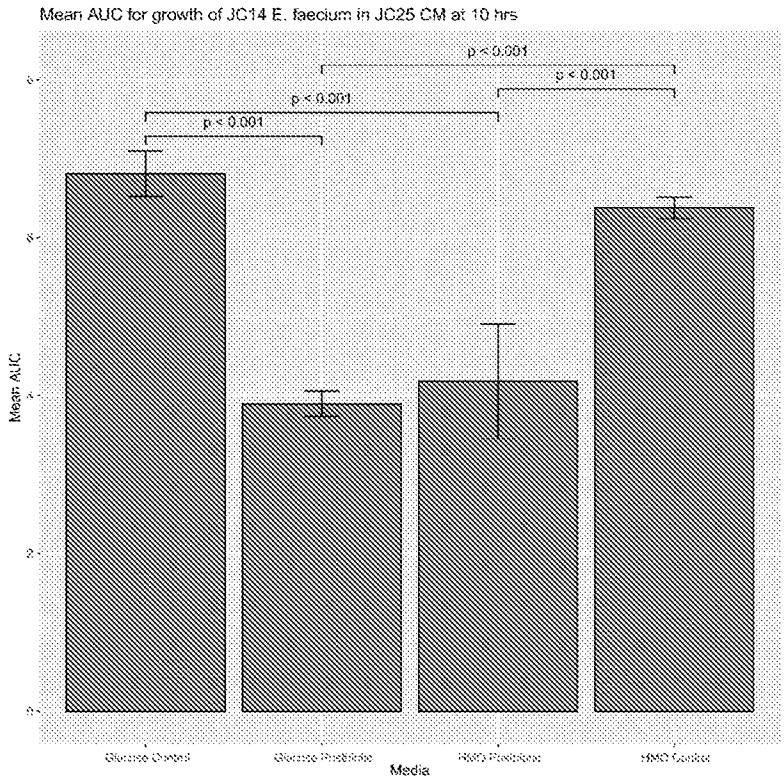

Mean AUC for growth of JC14 E. faecium in JC25 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 6B

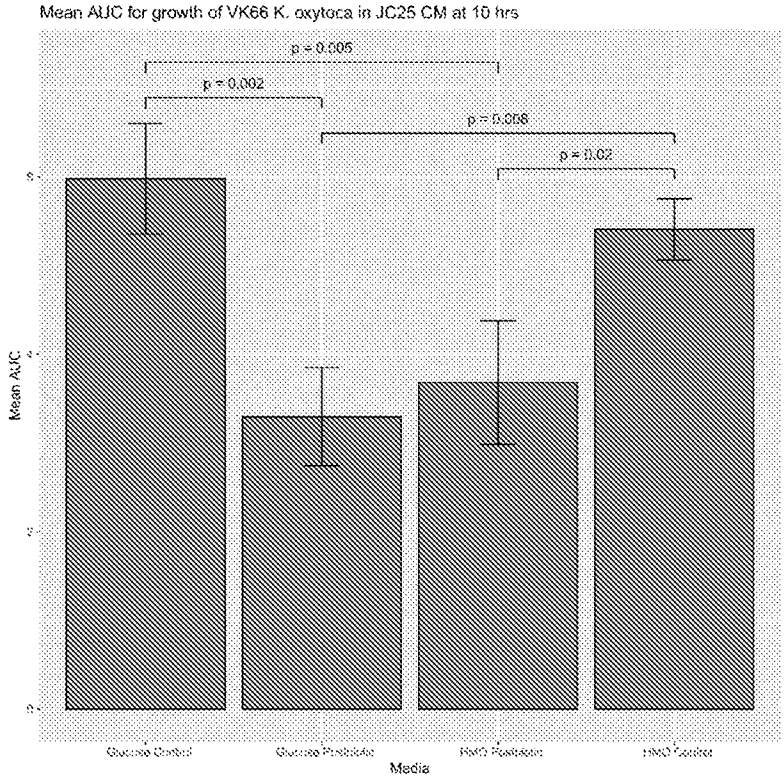

Mean AUC for growth of VK66 K. oxytoca in JC25 CM at 10 hrs
p = 0.005
p = 0.002
p = 0.008
p = 0.02
Mean AUC
Media

FIG. 6C

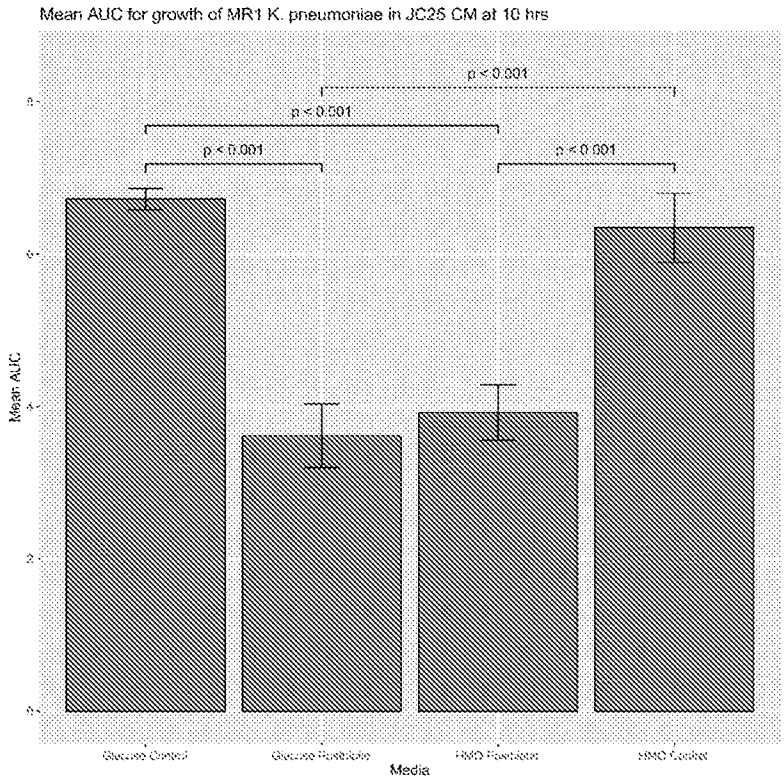

Mean AUC for growth of MR1 K. pneumoniae in JC25 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 6D

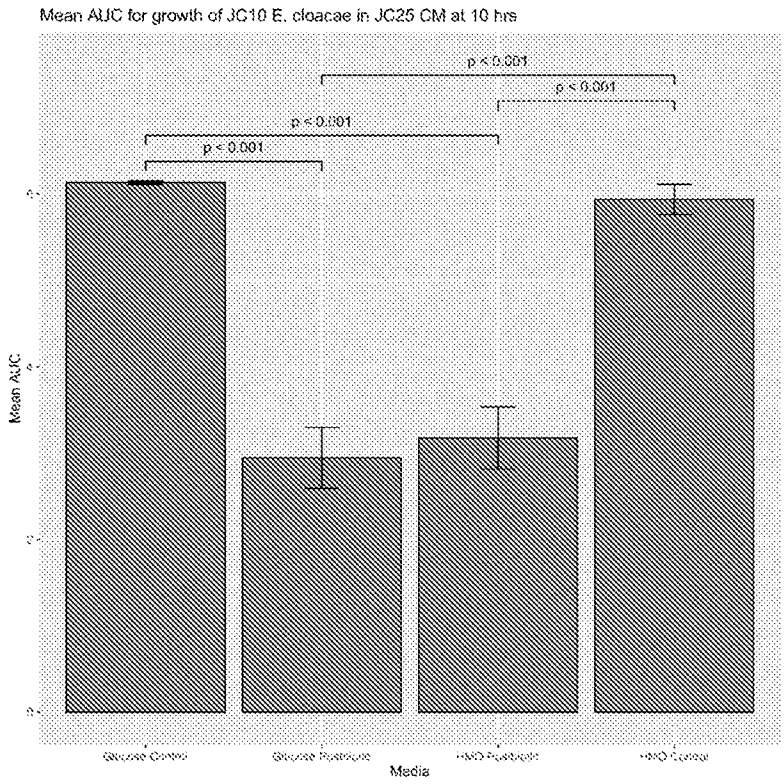

Mean AUC for growth of JC10 E. cloacae in JC25 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 6E

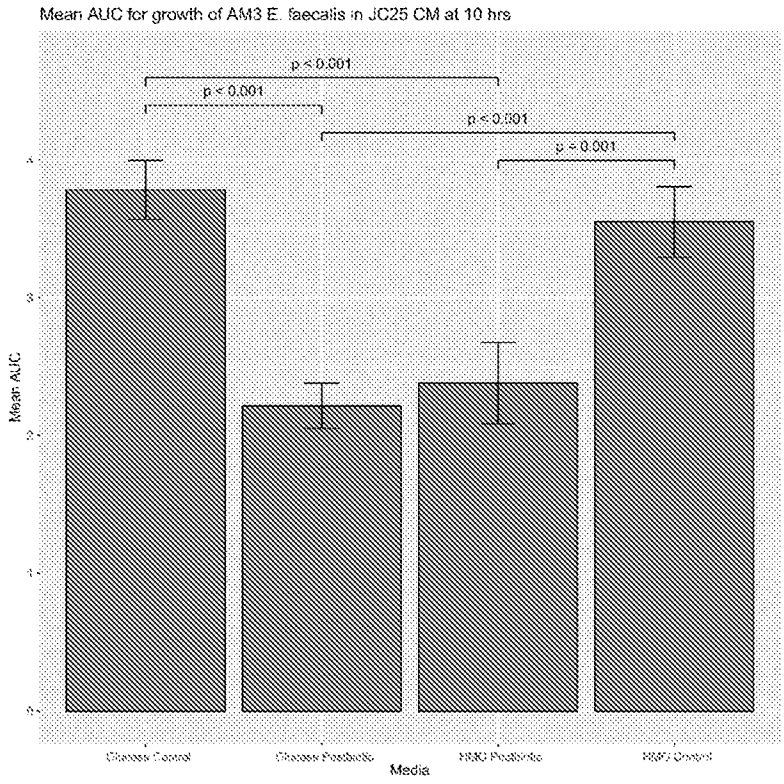

Mean AUC for growth of AM3 E. faecalis in JC25 CM at 10 hrs
p < 0.001
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 6F

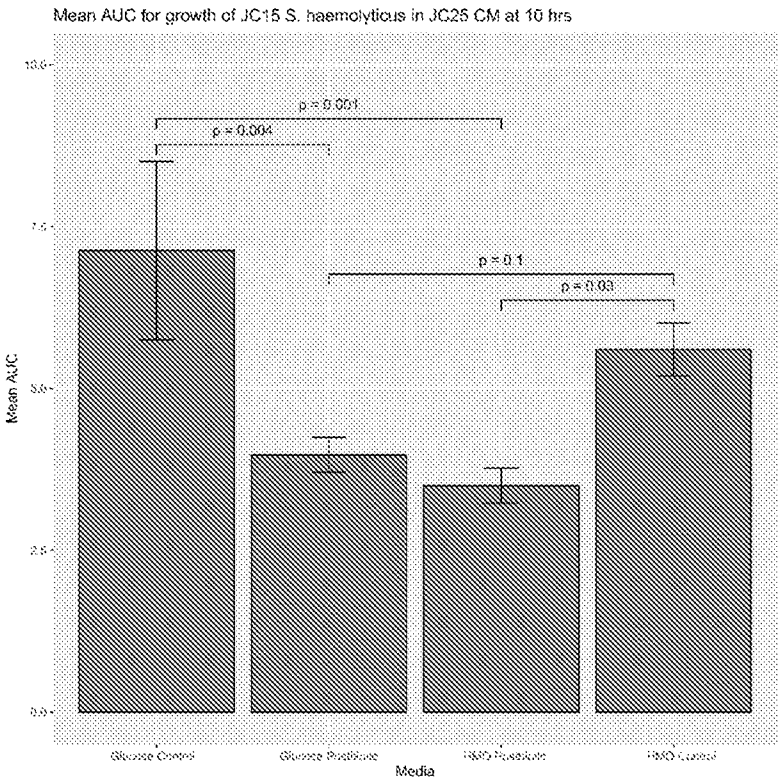

Mean AUC for growth of JC15 S. haemolyticus in JC25 CM at 16 hrs
p = 0.001
p = 0.004
p = 0.1
p = 0.03
Mean AUC
Media

FIG. 6G

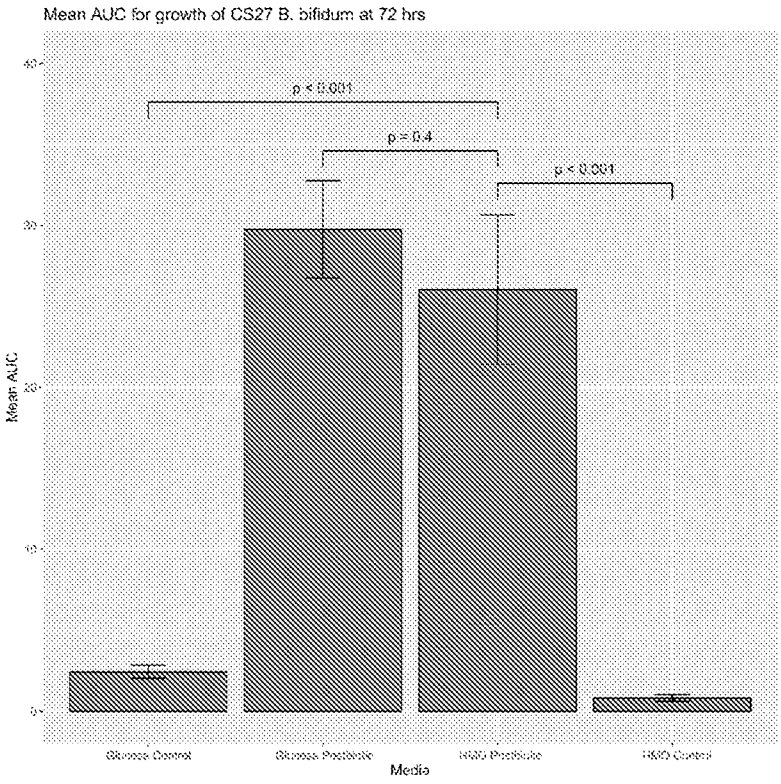

Mean AUC for growth of CS27 B. bifidum at 72 hrs
p < 0.001
p = 0.4
p < 0.001
Mean AUC
Media

FIG. 7A

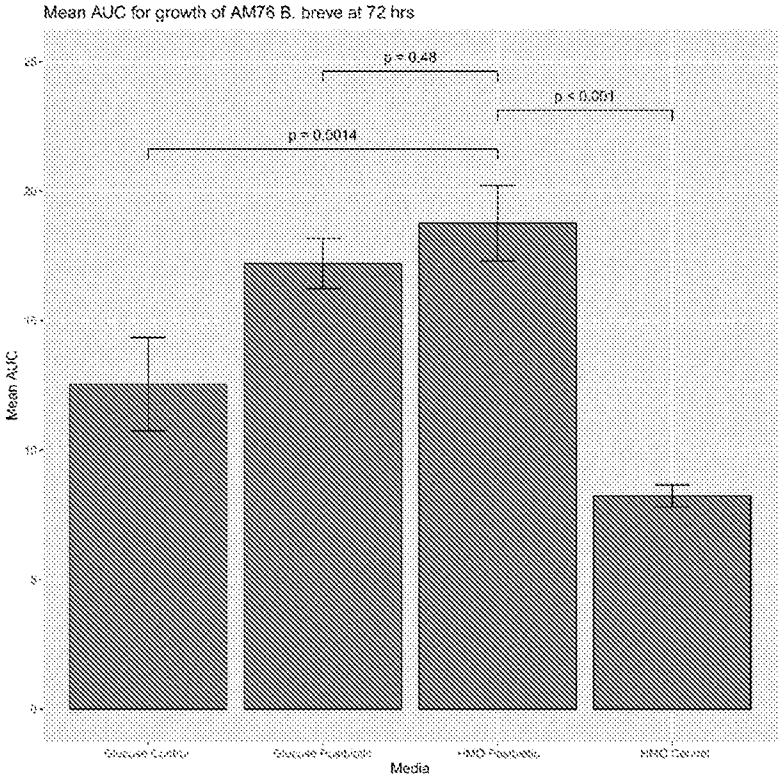

Mean AUC for growth of AM76 B. breve at 72 hrs
p = 0.48
p < 0.001
p = 0.0014
Mean AUC
Media

FIG. 7B

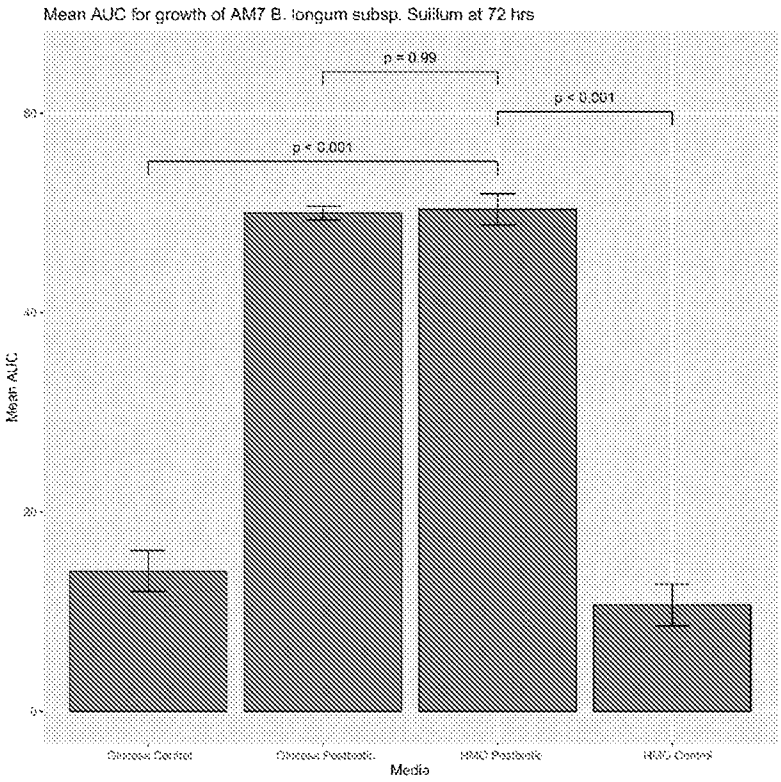

Mean AUC for growth of AM7 B. longum subsp. Suilum at 72 hrs
p = 0.99
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 7C

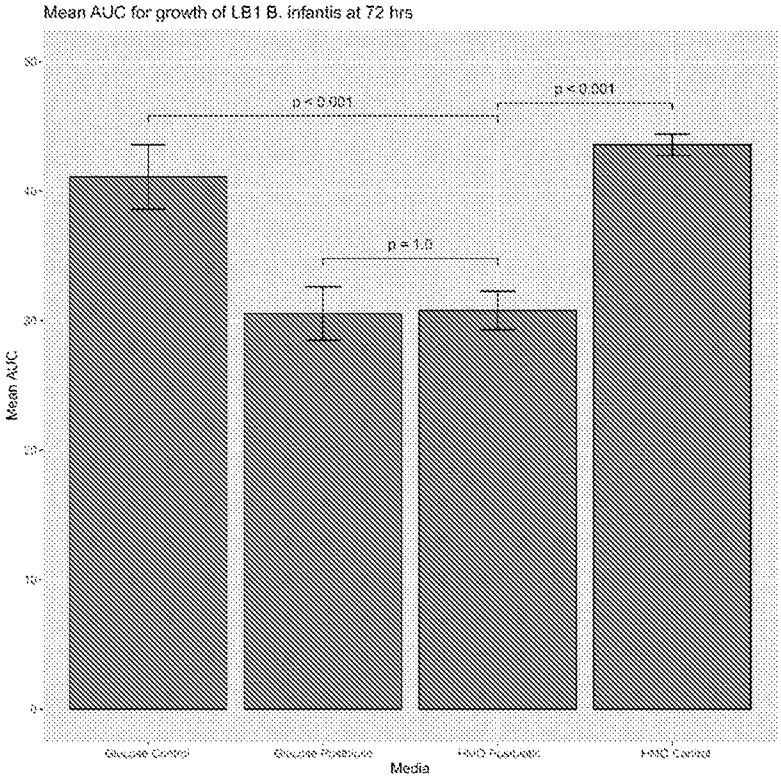

Mean AUC for growth of LB1 B. infantis at 72 hrs
p < 0.001
p < 0.001
p = 1.0
Mean AUC
Media

FIG. 7D

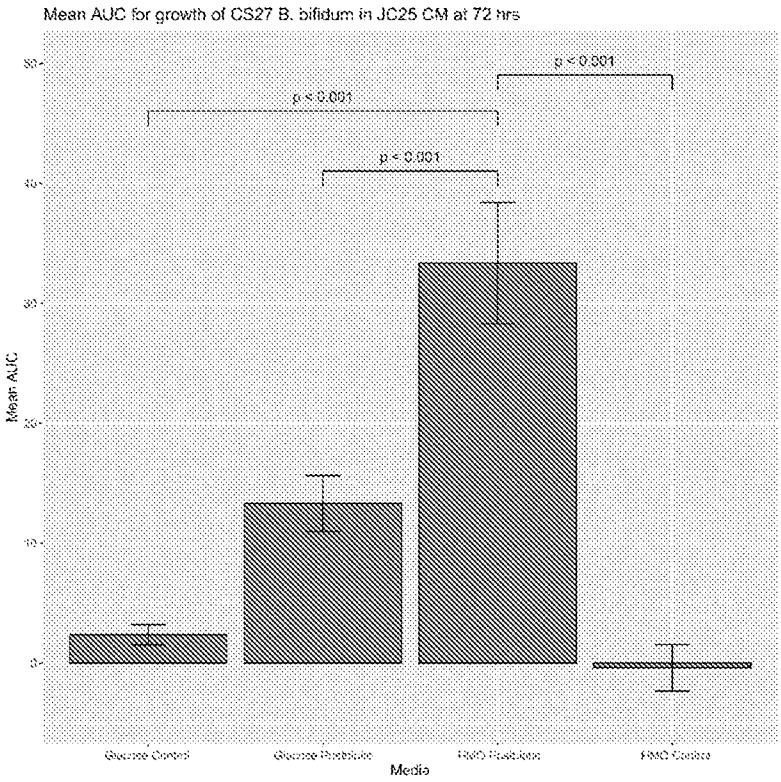

Mean AUC for growth of CS27 B. bifidum in JC25 CM at 72 hrs
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 8A

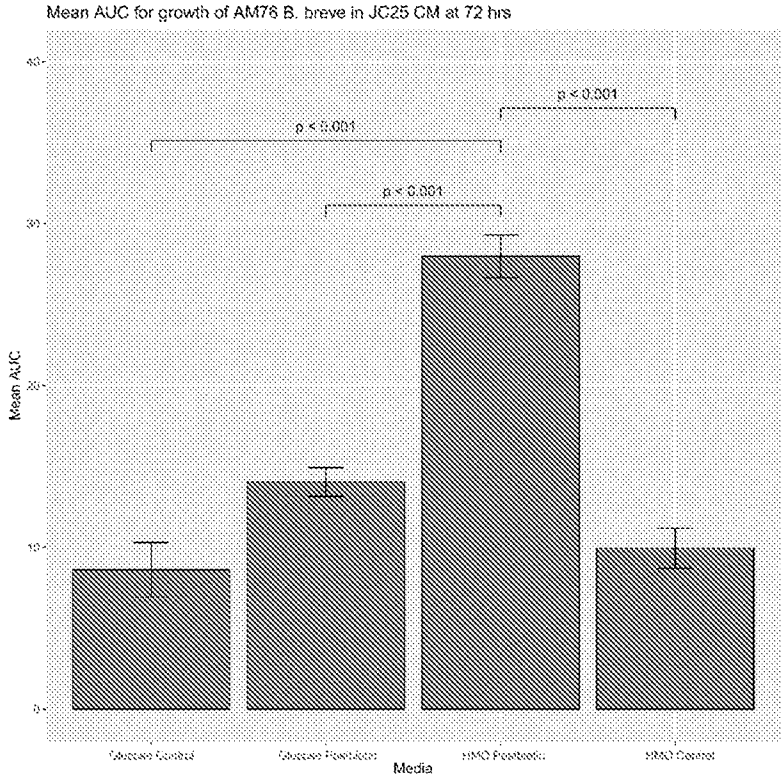

Mean AUC for growth of AM76 B. breve in JC25 CM at 72 hrs
p < 0.001
p < 0.001
p < 0.001
Mean AUC
Media

FIG. 8B

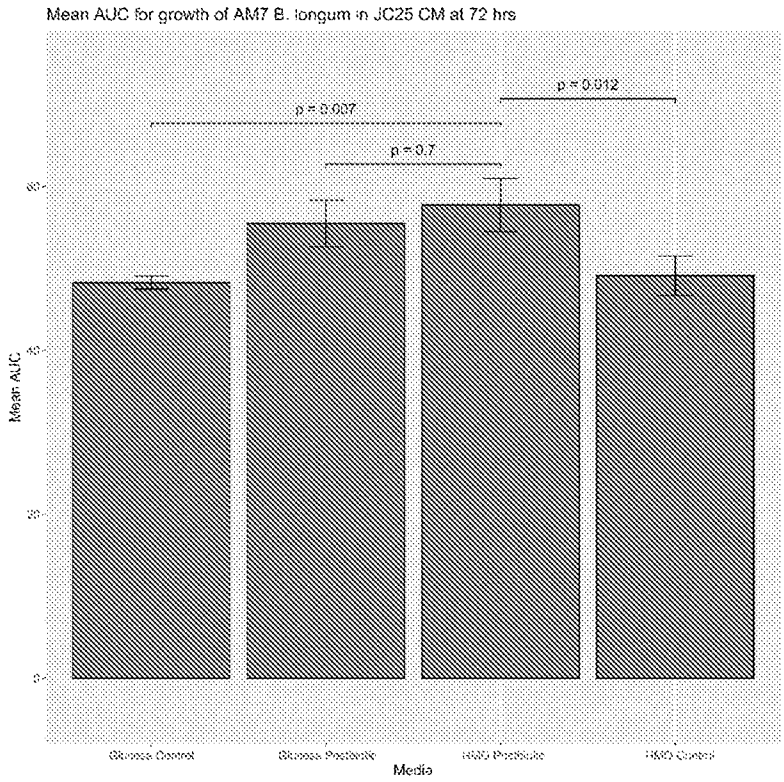

Mean AUC for growth of AM7 B. longum in JC25 CM at 72 hrs
p = 0.012
p = 0.007
p = 0.7
Mean AUC
Media

FIG. 8C

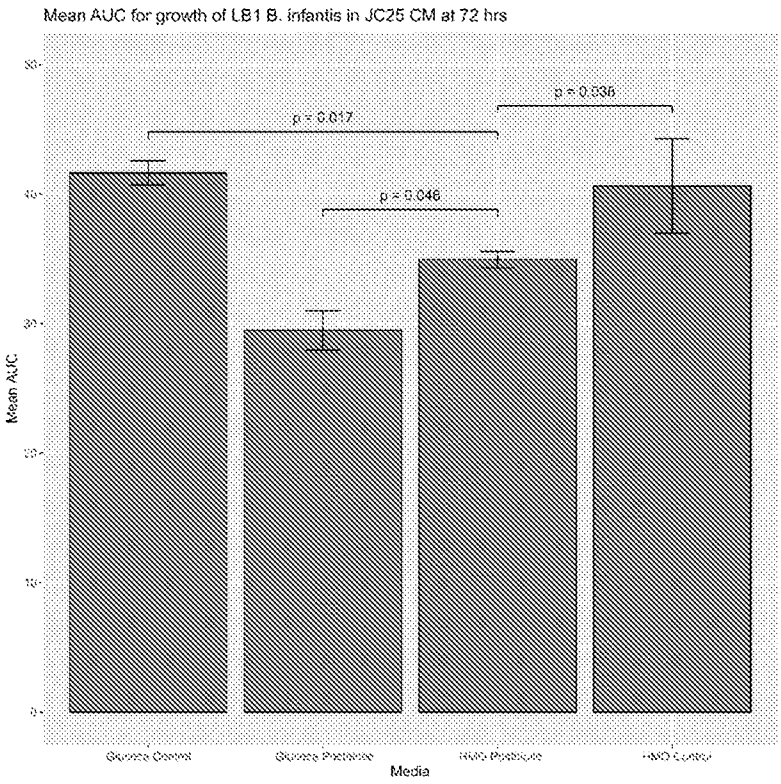

Mean AUC for growth of LB1 B. infantis in JC25 CM at 72 hrs
p = 0.017
p = 0.036
p = 0.046
Mean AUC
Media

FIG. 8D

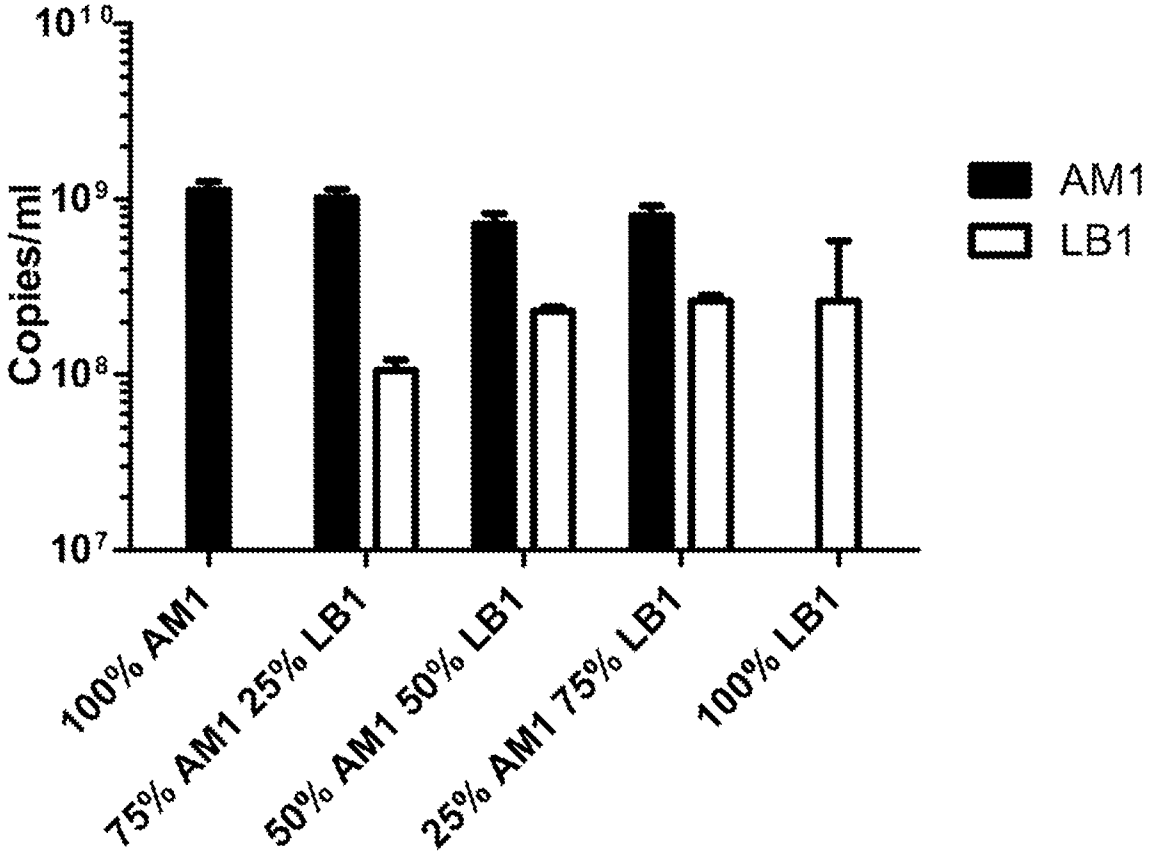

10^{10}
10^{9}
10^{8}
10^{7}
Copies/ml
AM1
LB1
100% AM1
75% AM1 25% LB1
50% AM1 50% LB1
25% AM1 75% LB1
100% LB1

FIG. 9

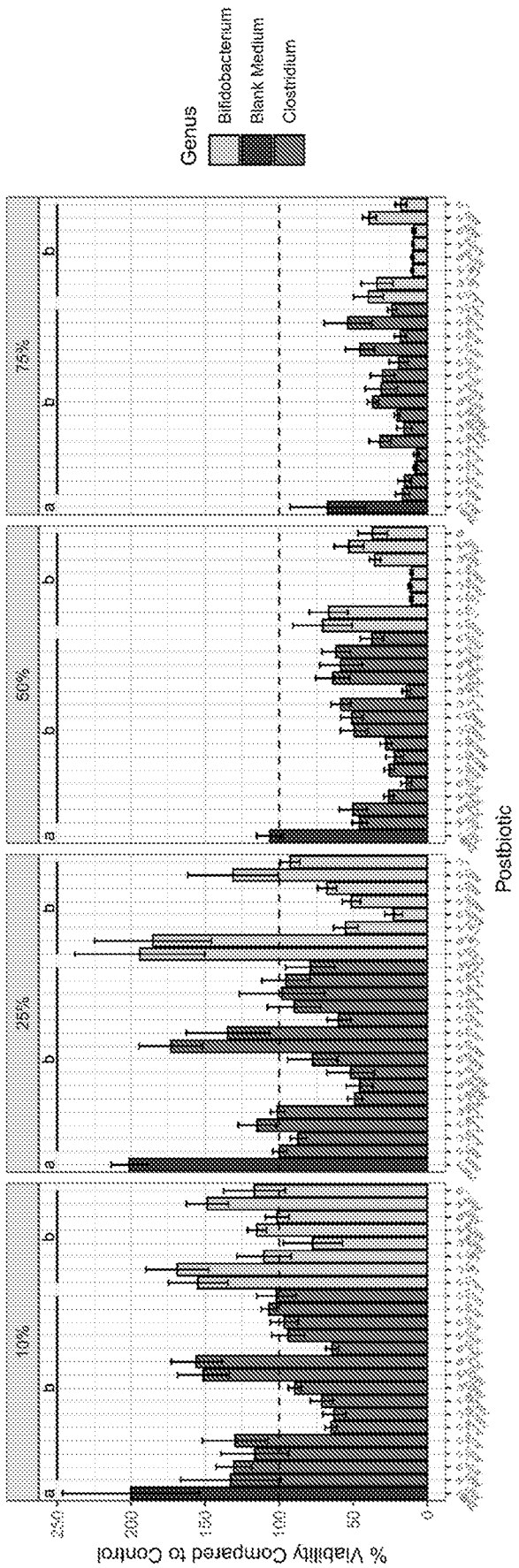

Genus
Bifidobacterium
Blank Medium
Clostridium
% Viability Compared to Control
250
200
150
100
50
0
10%
25%
50%
75%
a
b
Postbiotic

FIG. 10

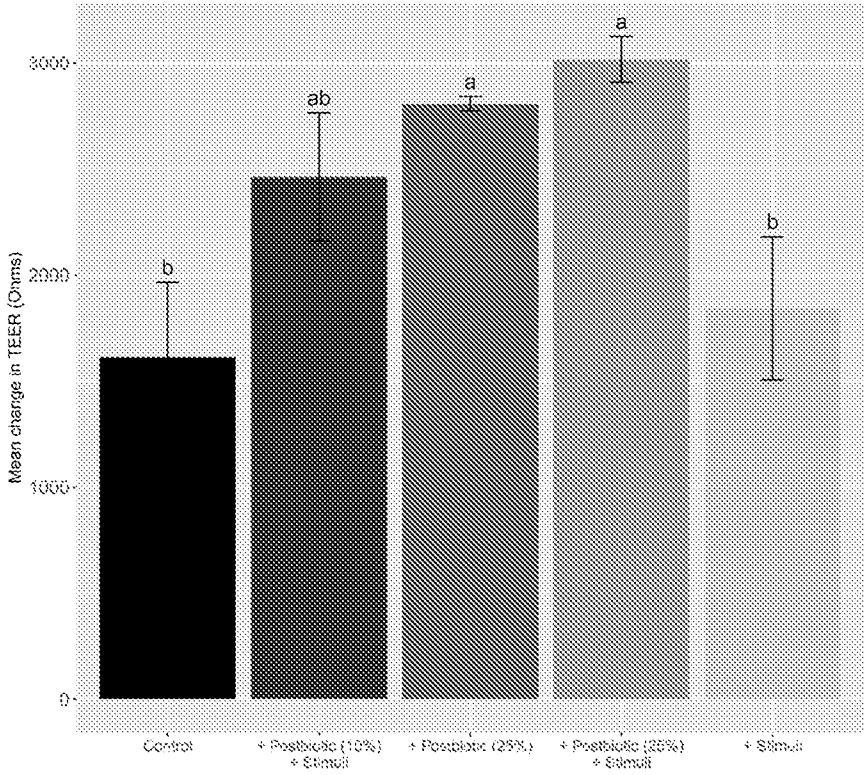


FIG. 11A

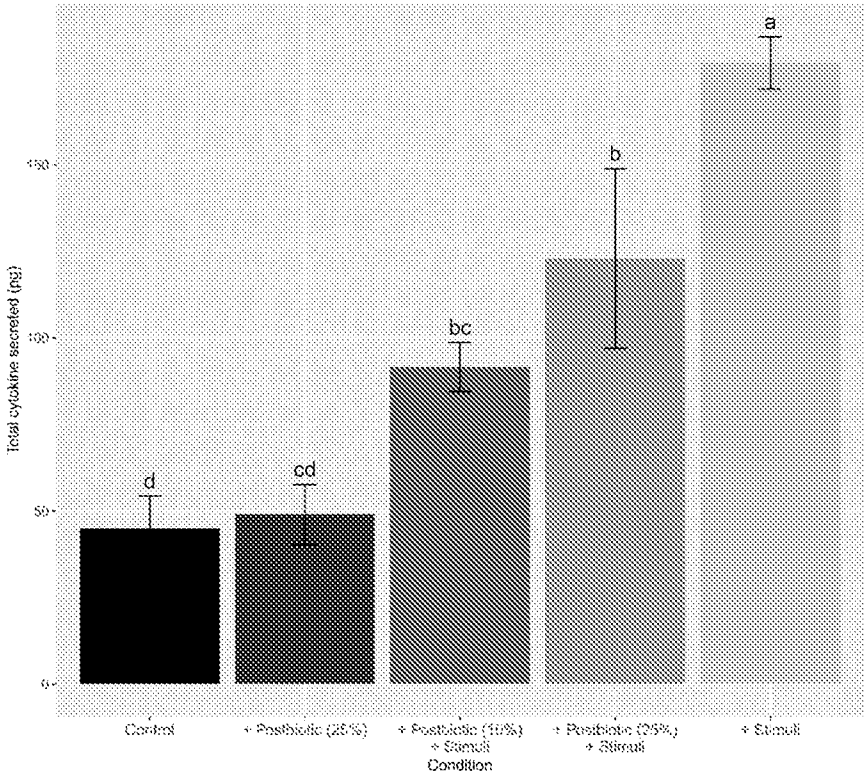


FIG. 11B

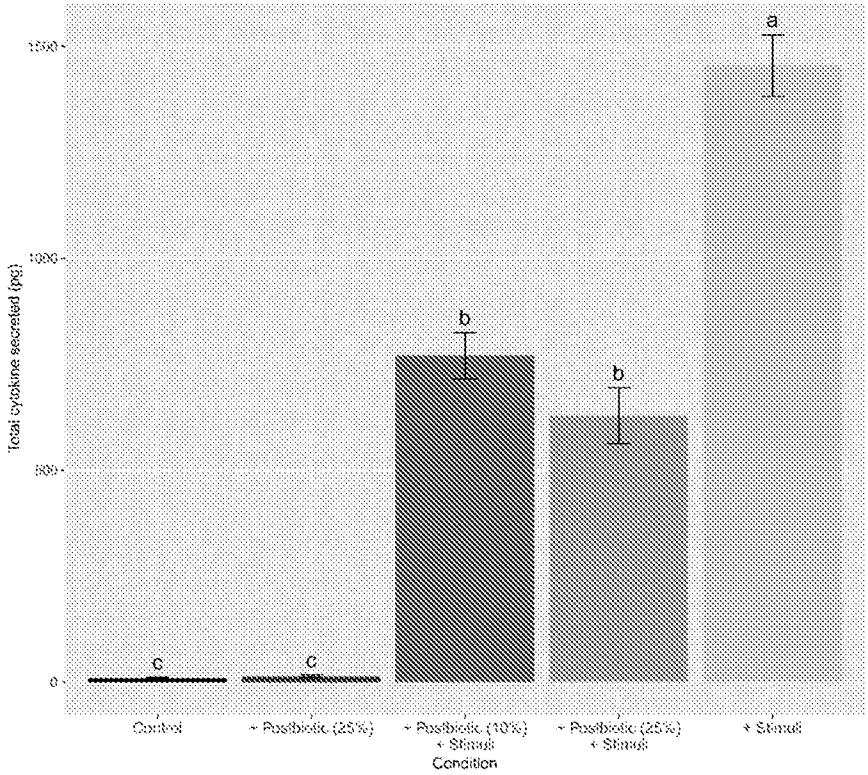


FIG. 11C

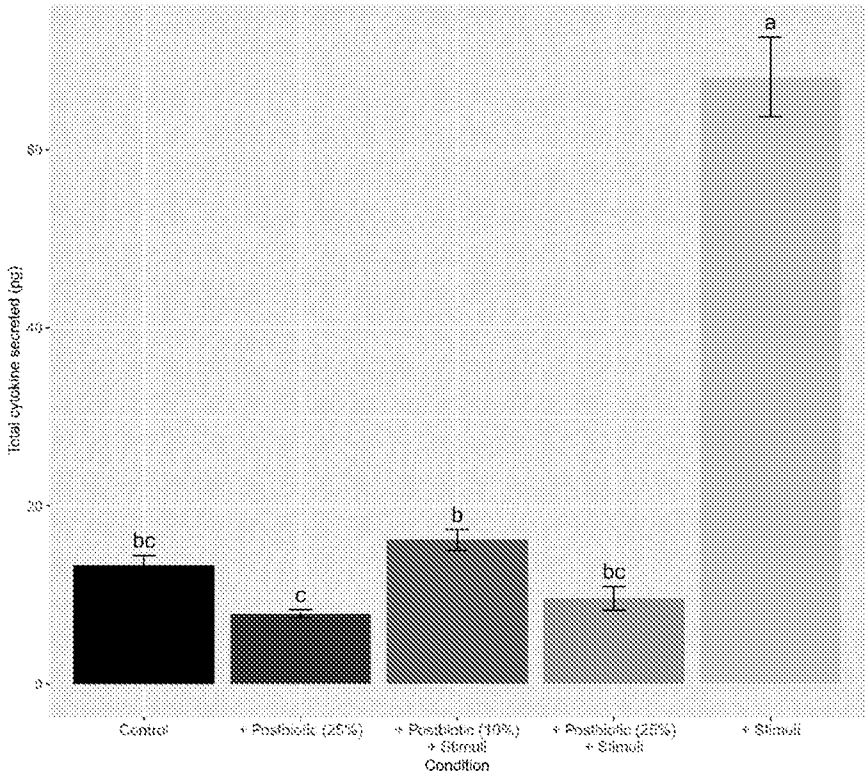


FIG. 11D

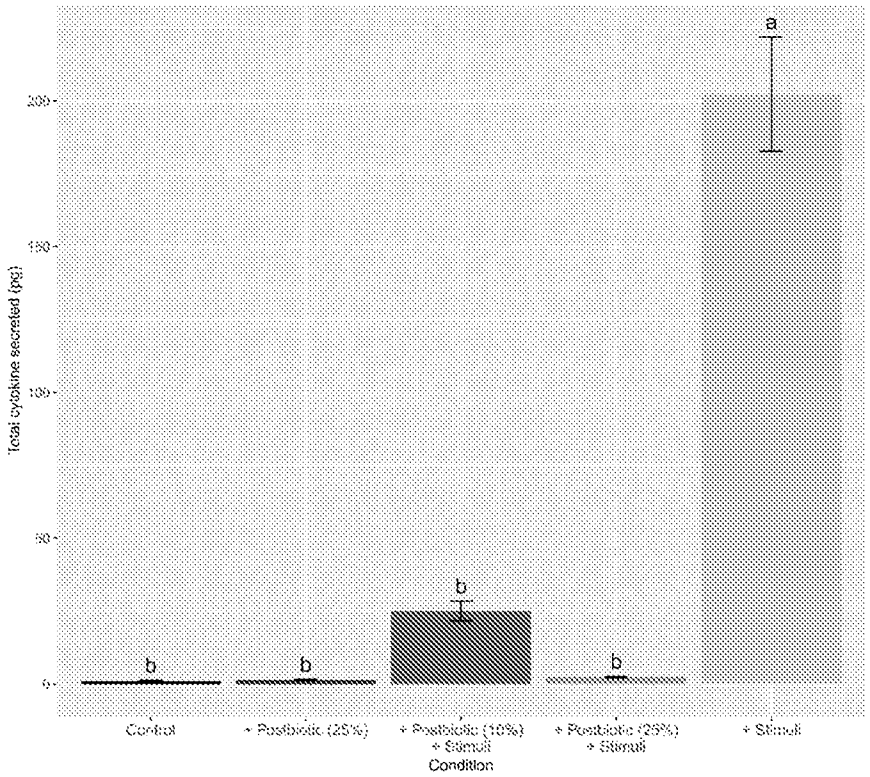


FIG. 11E

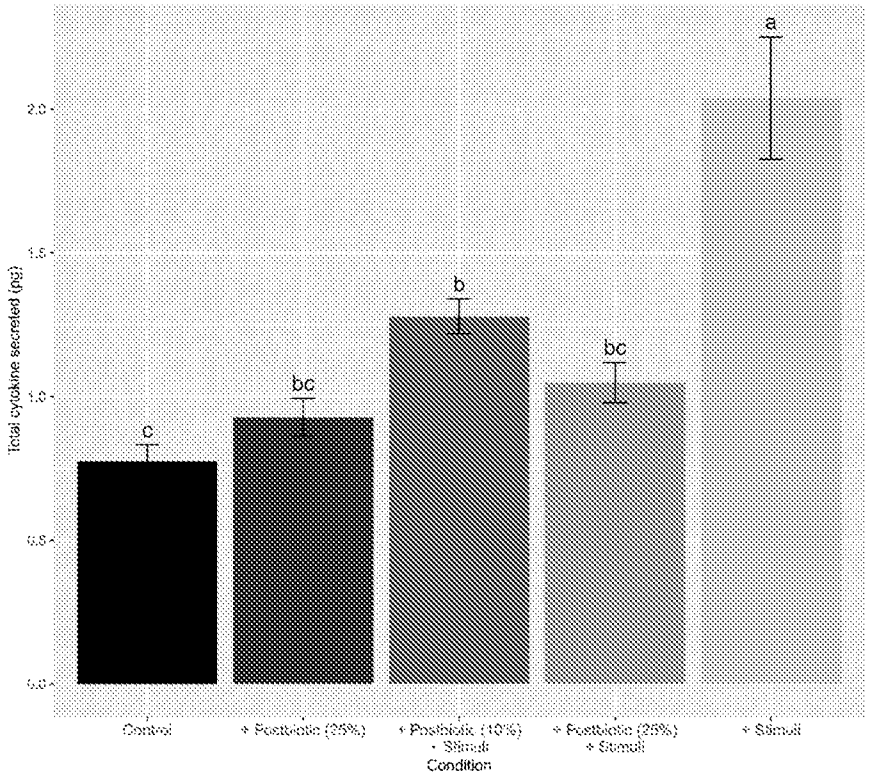


FIG. 11F

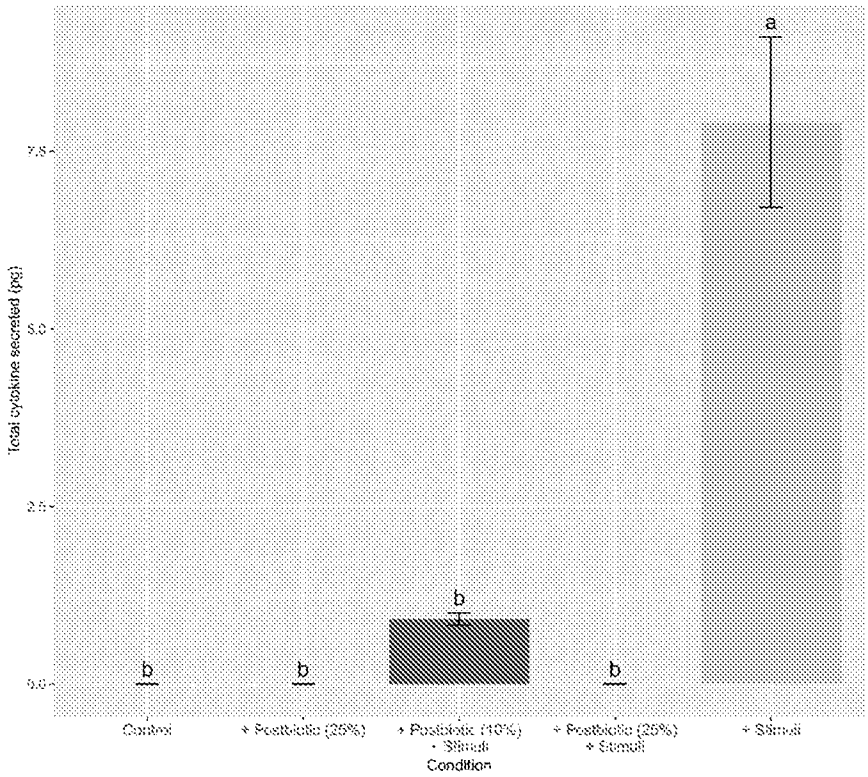


FIG. 11G

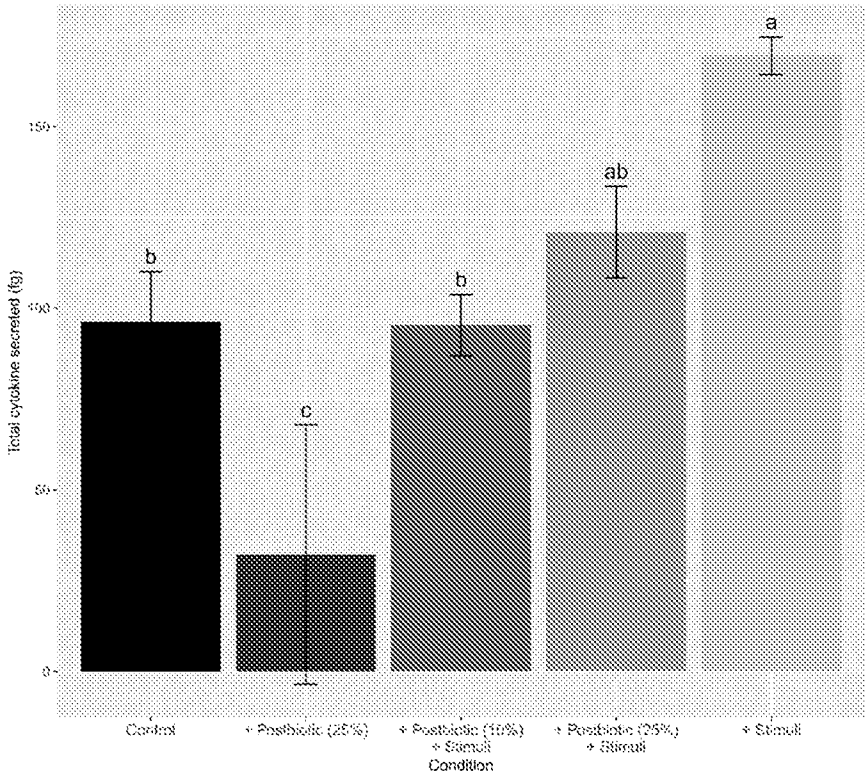


FIG. 11H

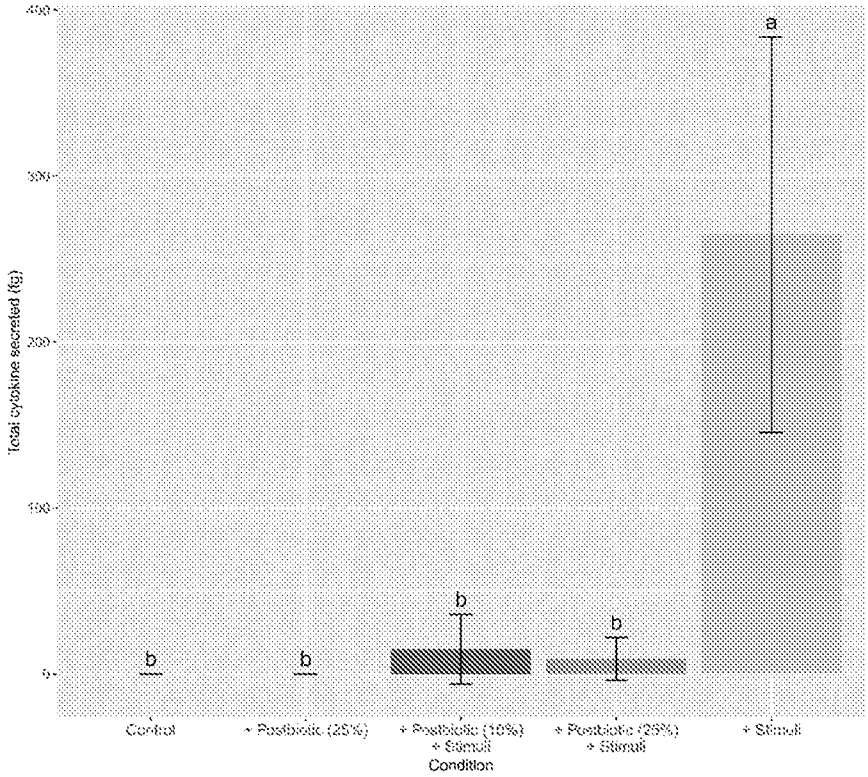


FIG. 11I

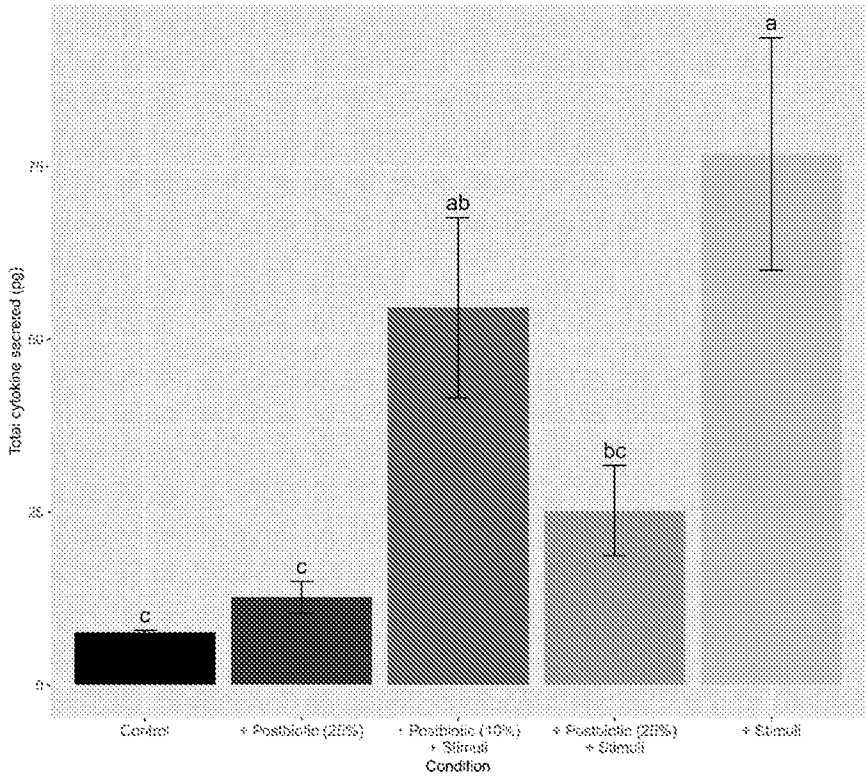


FIG. 11J

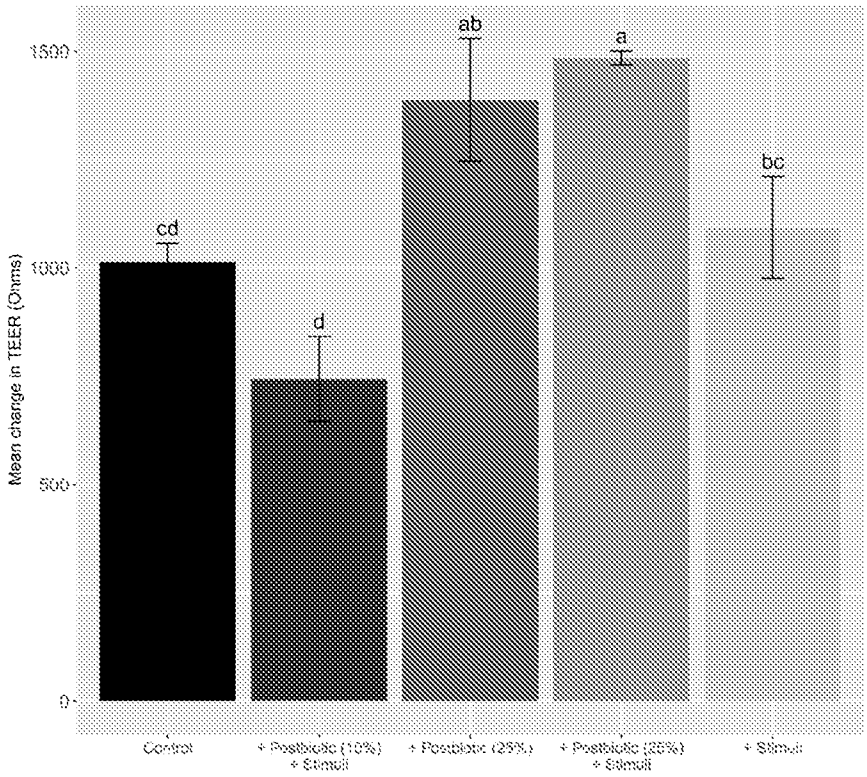


FIG. 12A

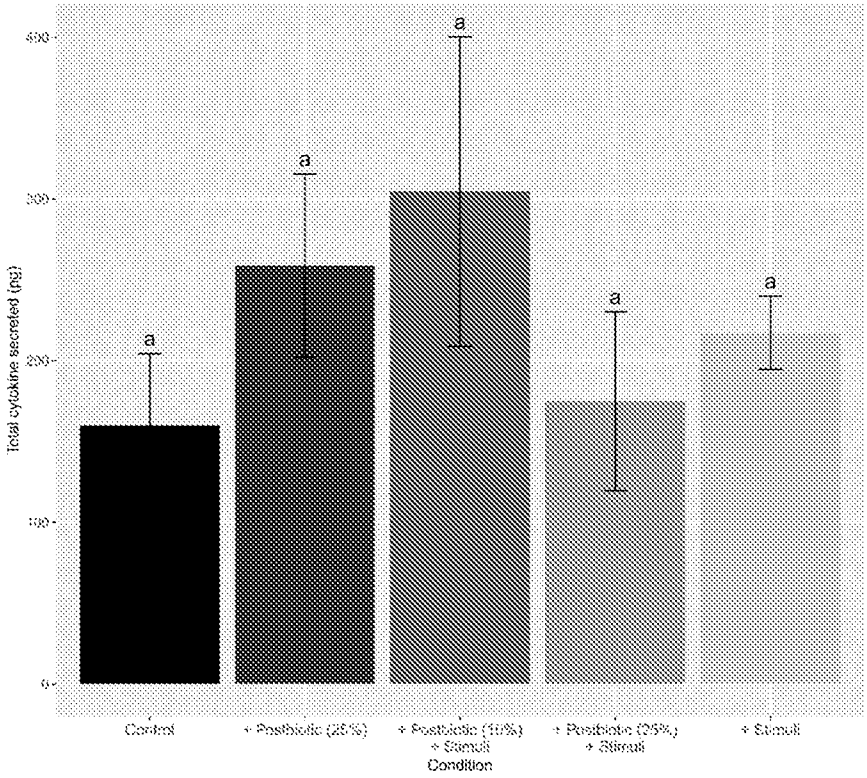


FIG. 12B

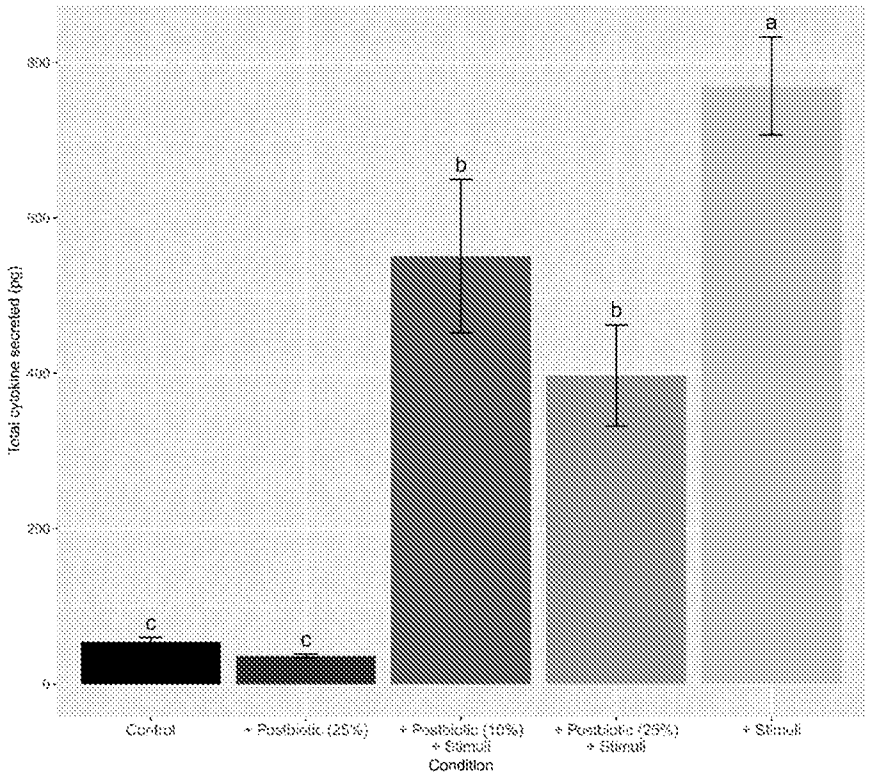


FIG. 12C

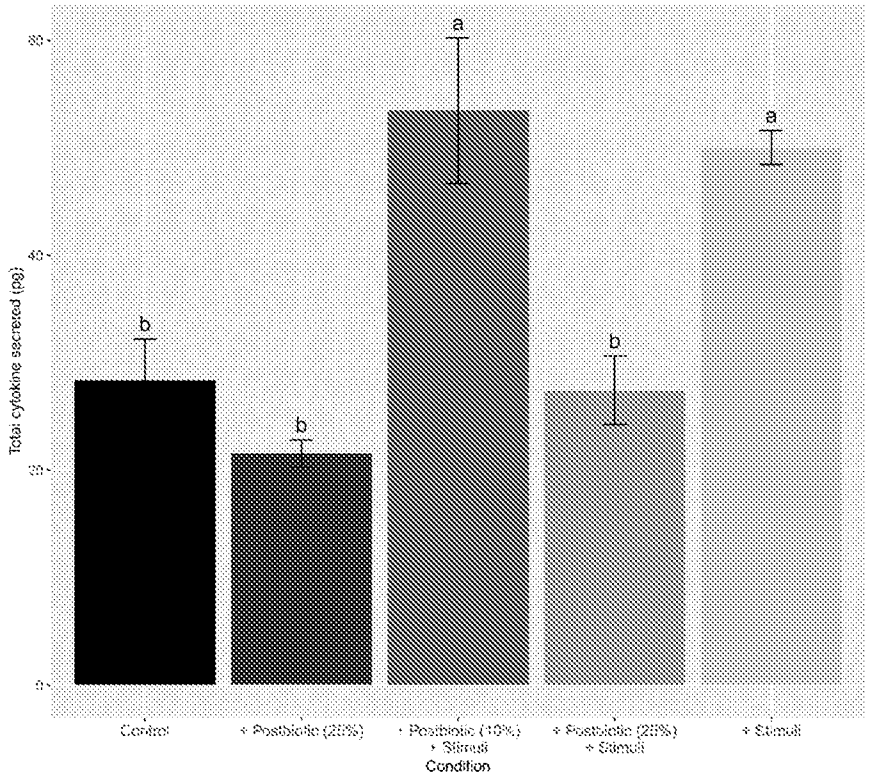


FIG. 12D

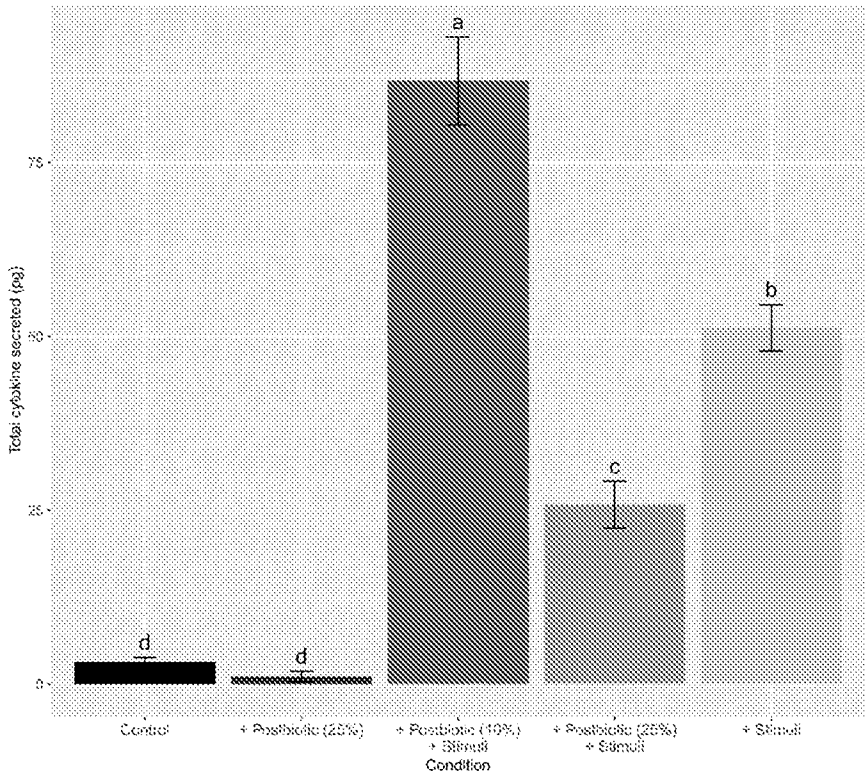


FIG. 12E

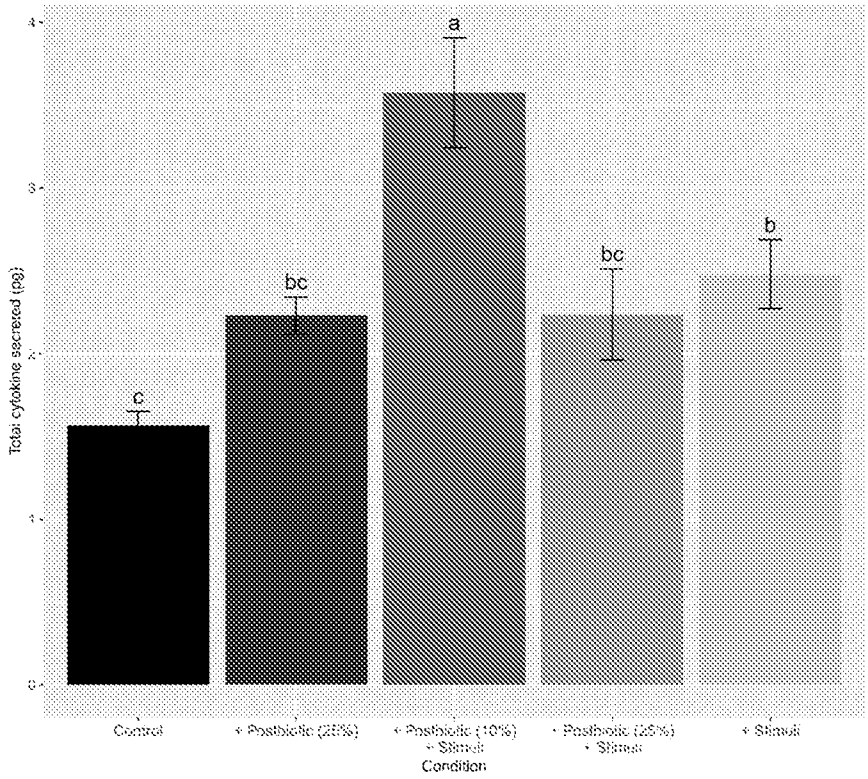


FIG. 12F

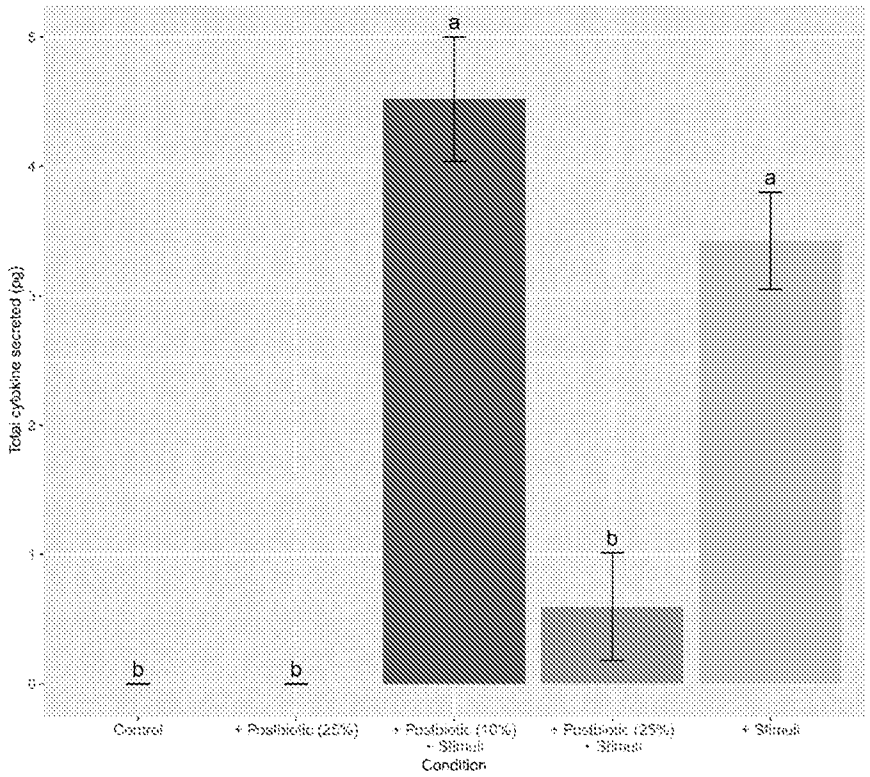


FIG. 12G

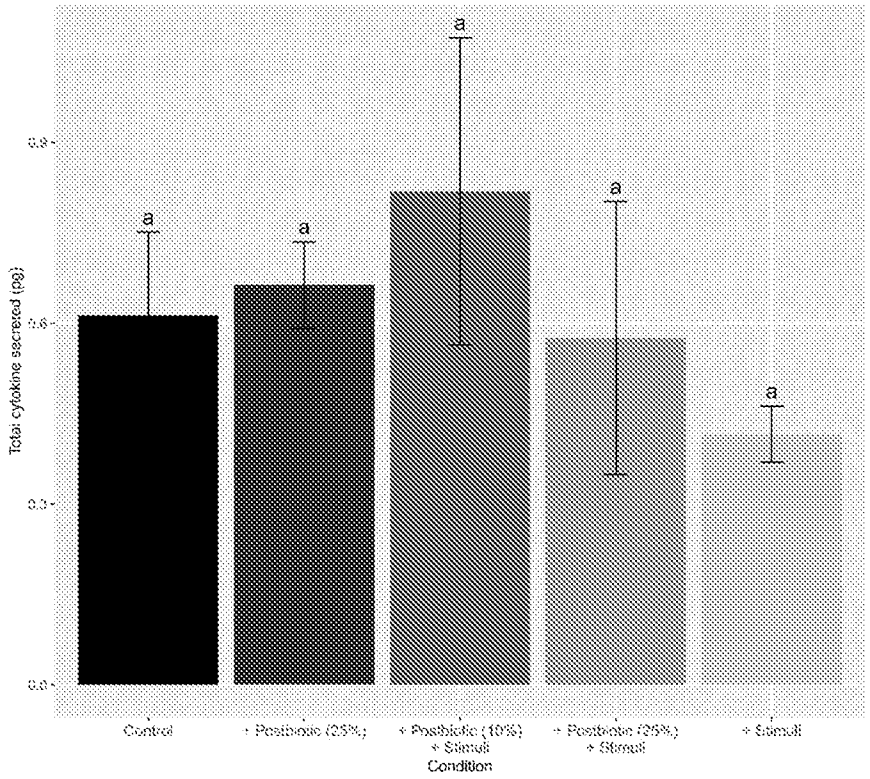


FIG. 12H

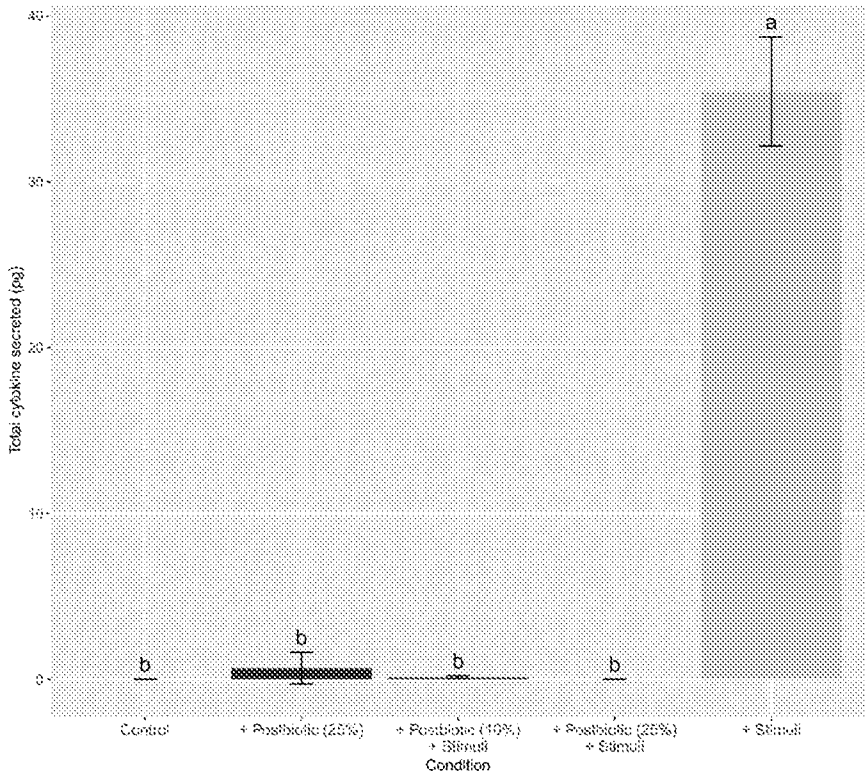


FIG. 12I

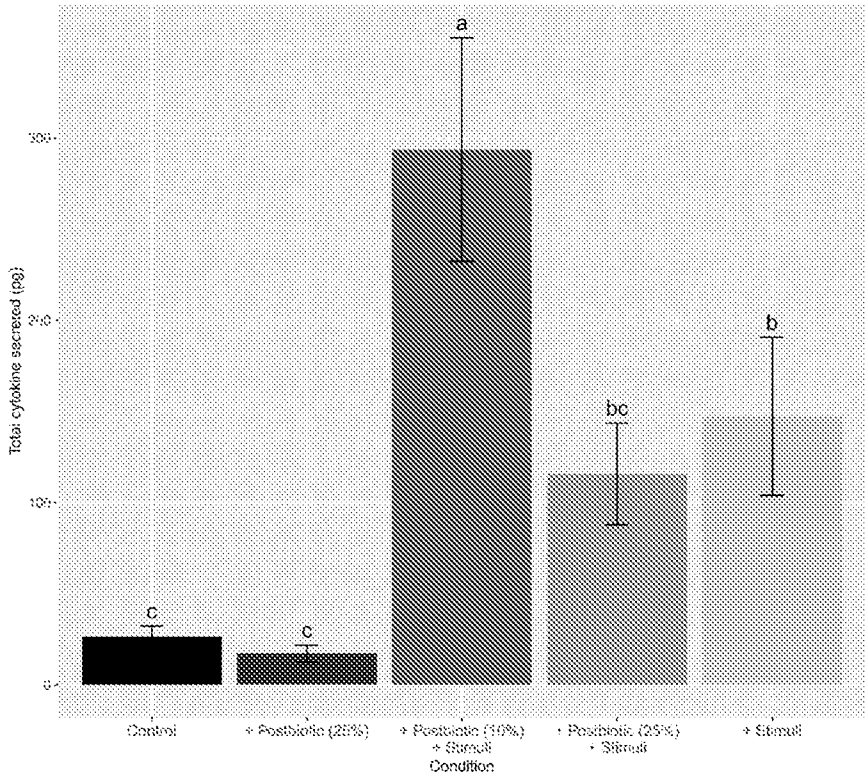


FIG. 12J

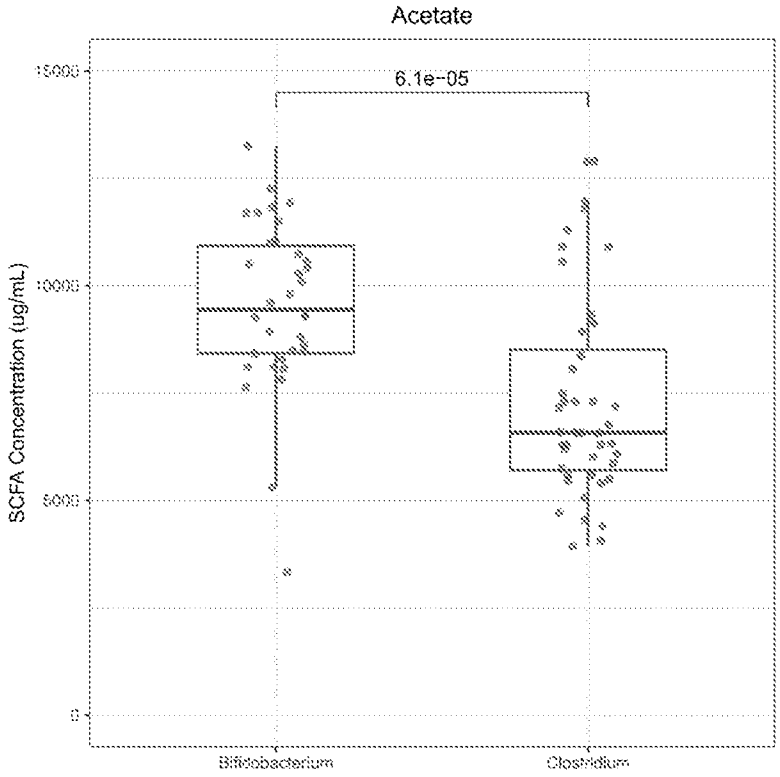


FIG. 13A

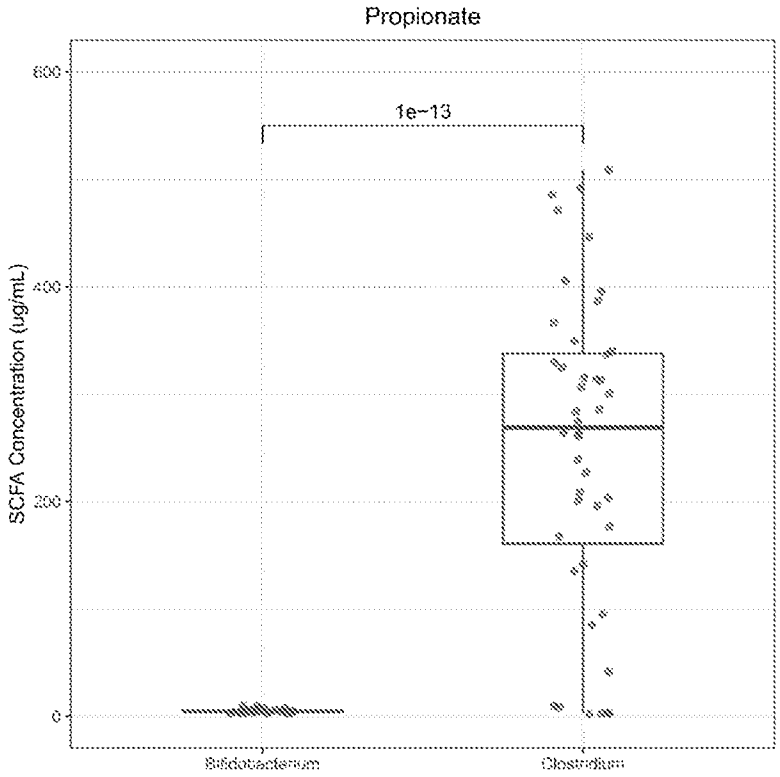


FIG. 13B

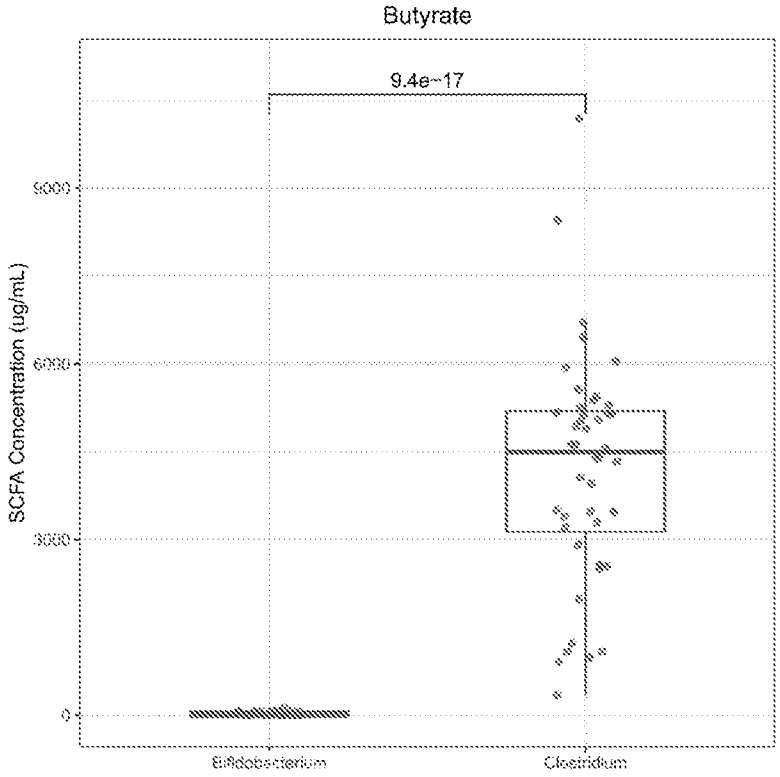


FIG. 13C

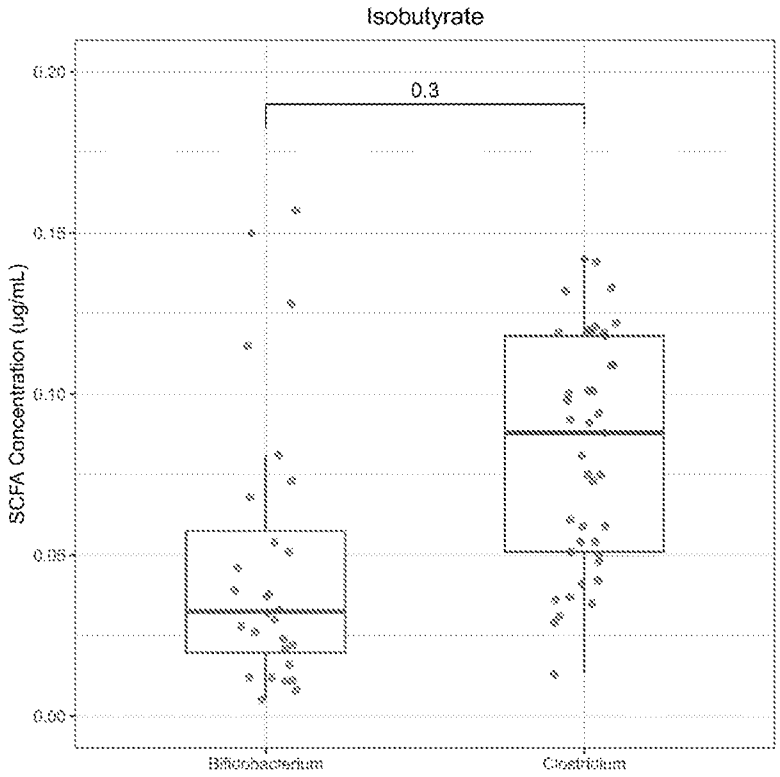


FIG. 13D

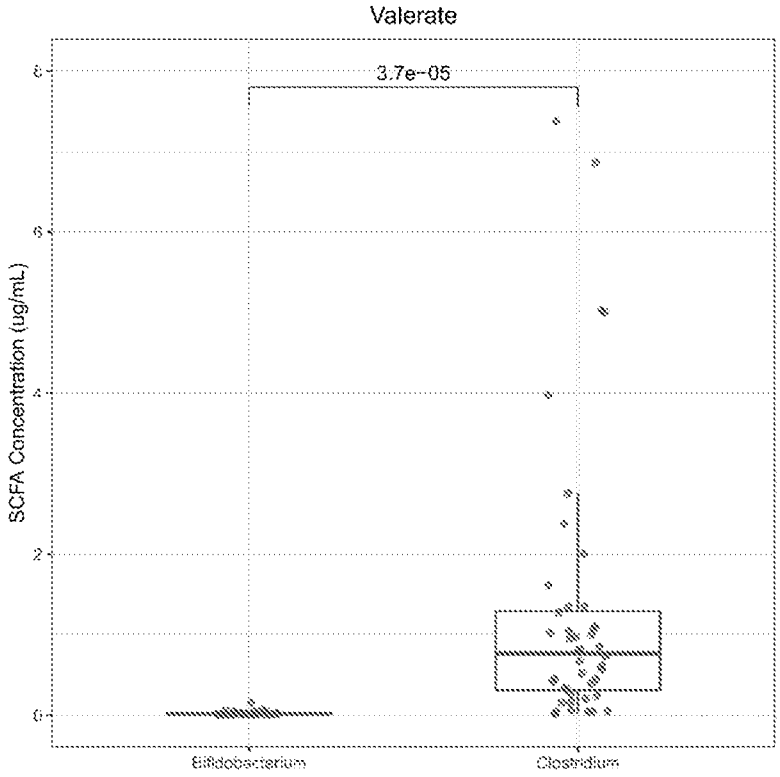


FIG. 13E

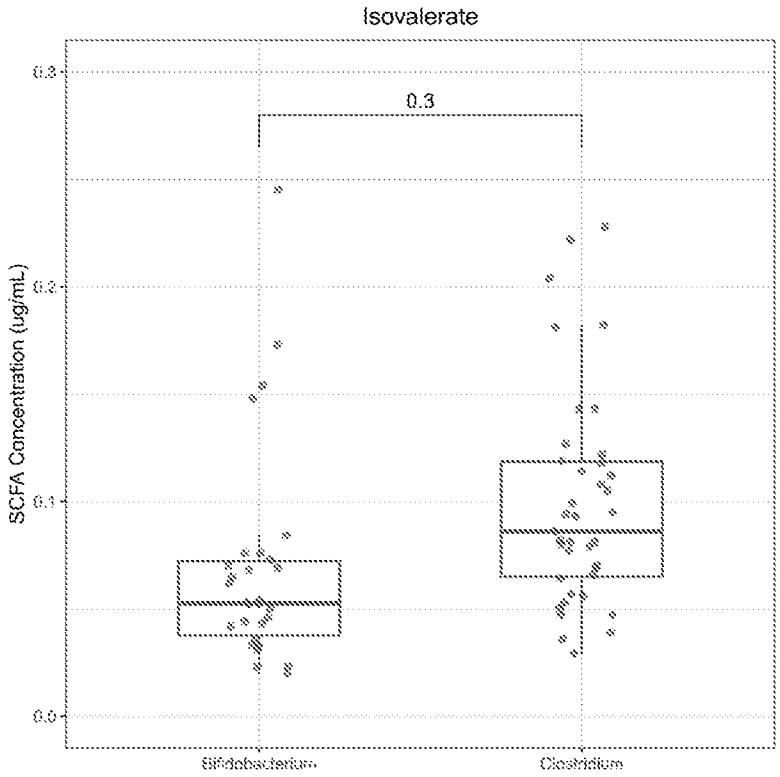


FIG. 13F

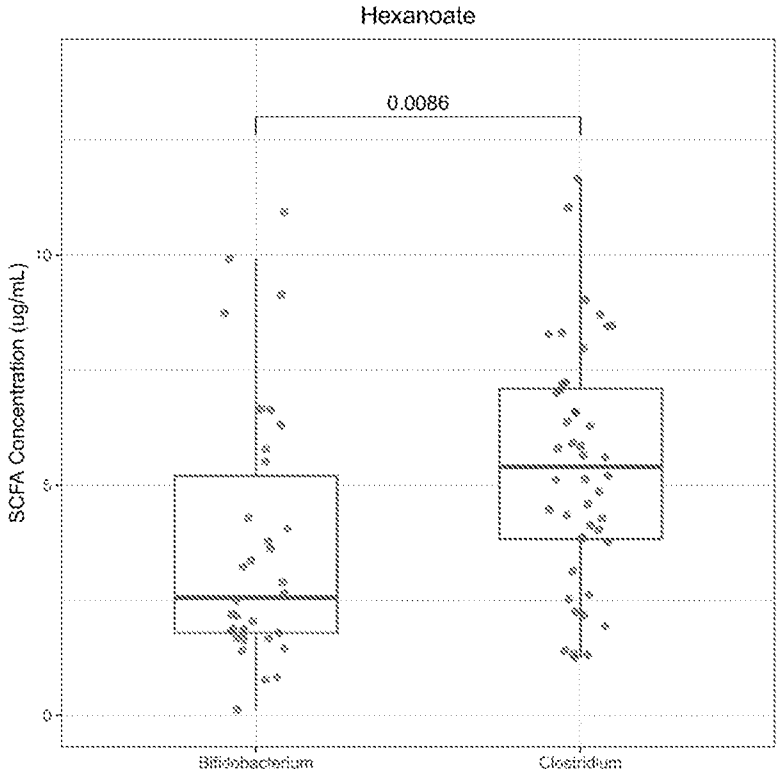


FIG. 13G

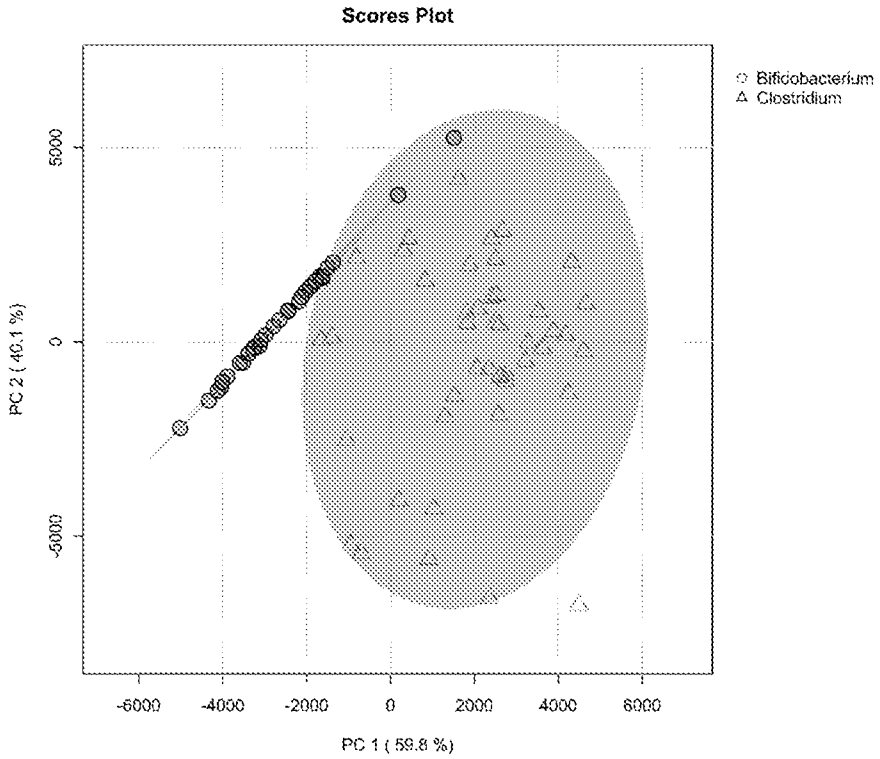


FIG. 13H

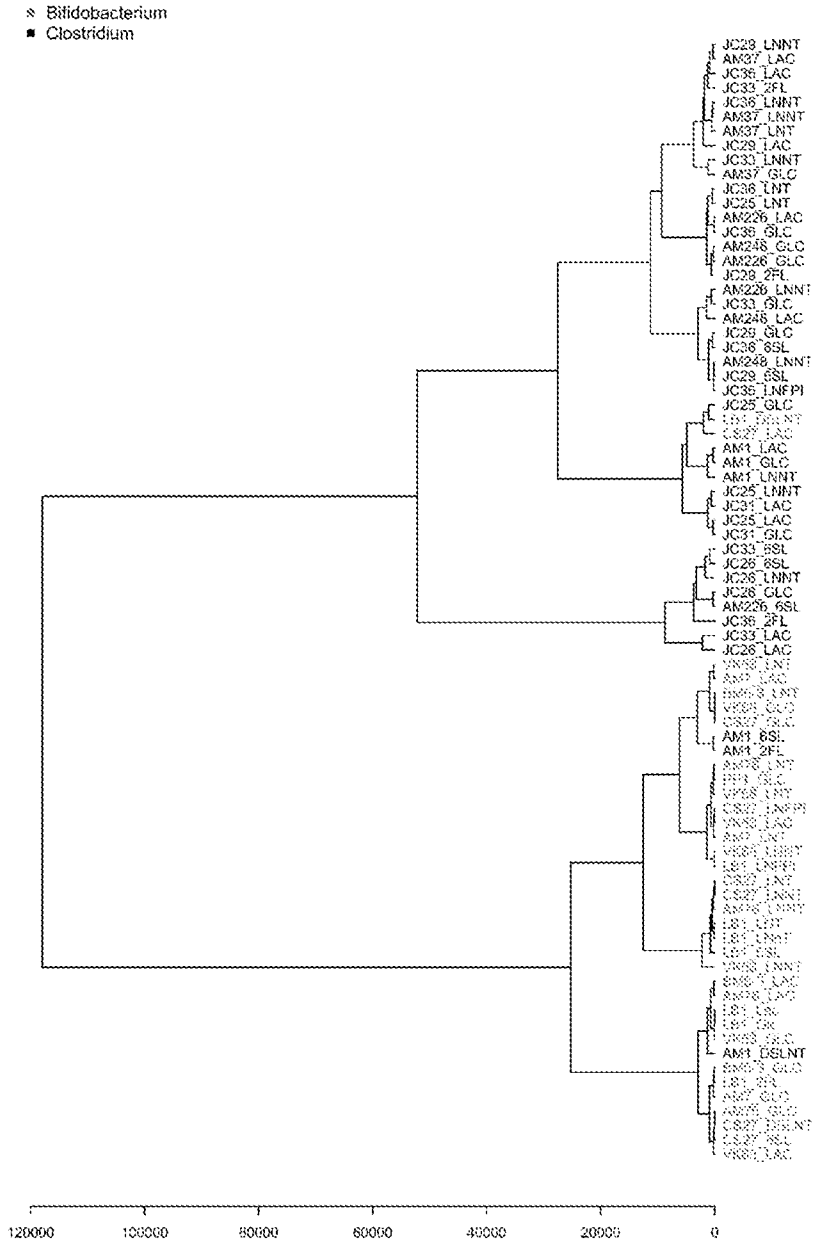

Bifidobacterium
Clostridium
JC29_LNNT
AM37_LAC
JC36_LAC
JC33_2FL
JC36_LNNT
AM37_LNNT
AM37_LNT
JC29_LAC
JC33_LNNT
AM37_GLC
JC36_LNT
JC25_LNT
AM226_LAC
JC36_GLC
AM248_GLC
AM226_GLC
JC29_2FL
AM226_LNNT
JC33_GLC
AM248_LAC
JC29_GLC
JC36_6SL
AM248_LNNT
JC29_6SL
JC36_LNFPI
JC25_GLC
AM1_LAC
AM1_GLC
AM1_LNNT
JC25_LNNT
JC31_LAC
JC25_LAC
JC31_GLC
JC33_6SL
JC26_6SL
JC26_LNNT
JC26_GLC
AM226_6SL
JC36_2FL
JC33_LAC
JC26_LAC
AM1_6SL
AM1_2FL
AM1_DSLNT
120000
100000
80000
60000
40000
20000
0

FIG. 13I

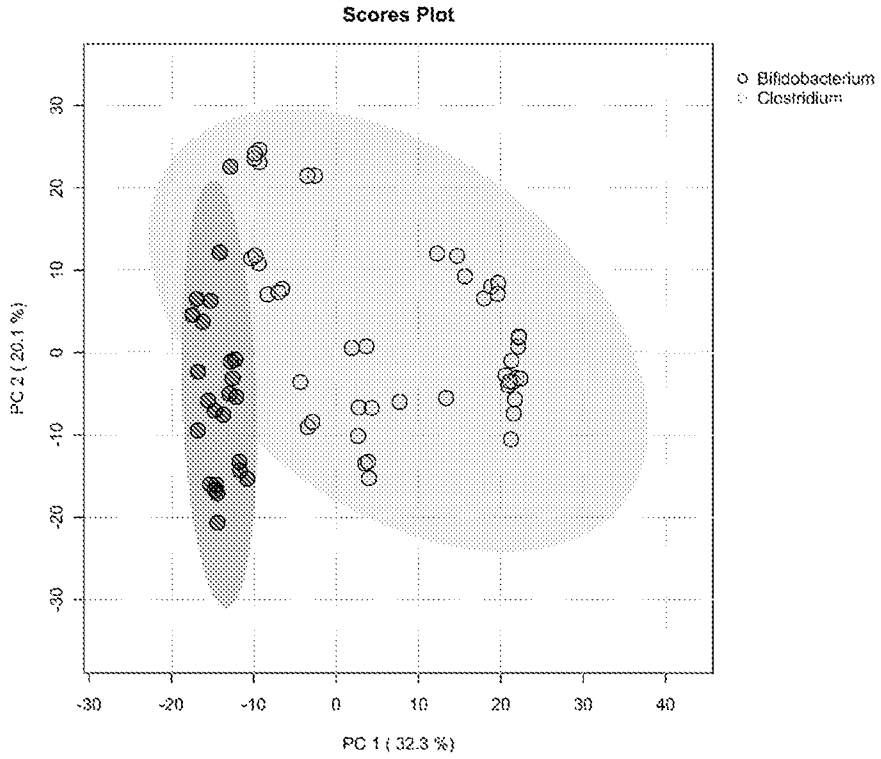

Scores Plot
Bifidobacterium
Clostridium
PC 2 (20.1 %)
PC 1 (32.3 %)
30
20
10
0
-10
-20
-30
-30
-20
-10
0
10
20
30
40

FIG. 14A

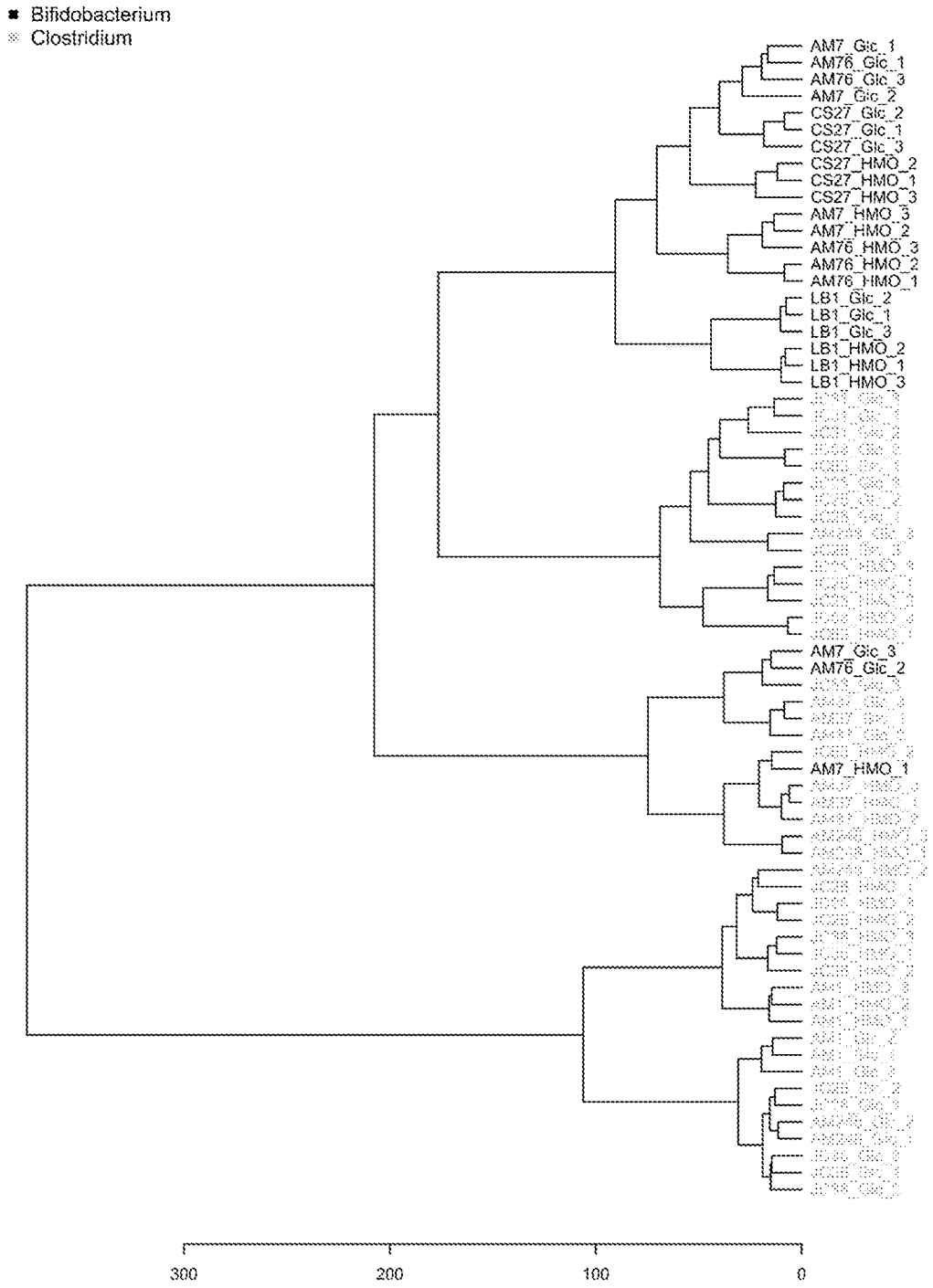

Bifidobacterium
Clostridium
AM7_Glc_1
AM76_Glc_1
AM76_Glc_3
AM7_Glc_2
CS27_Glc_2
CS27_Glc_1
CS27_Glc_3
CS27_HMO_2
CS27_HMO_1
CS27_HMO_3
AM7_HMO_3
AM7_HMO_2
AM76_HMO_3
AM76_HMO_2
AM76_HMO_1
LB1_Glc_2
LB1_Glc_1
LB1_Glc_3
LB1_HMO_2
LB1_HMO_1
LB1_HMO_3
AM7_Glc_3
AM76_Glc_2
AM7_HMO_1
300
200
100
0

FIG. 14B

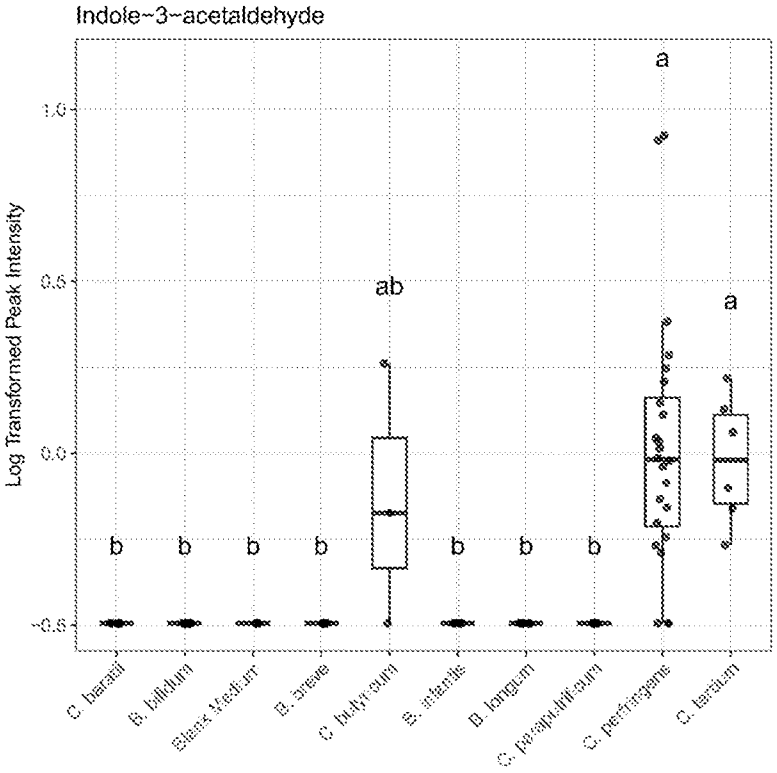


FIG. 15A

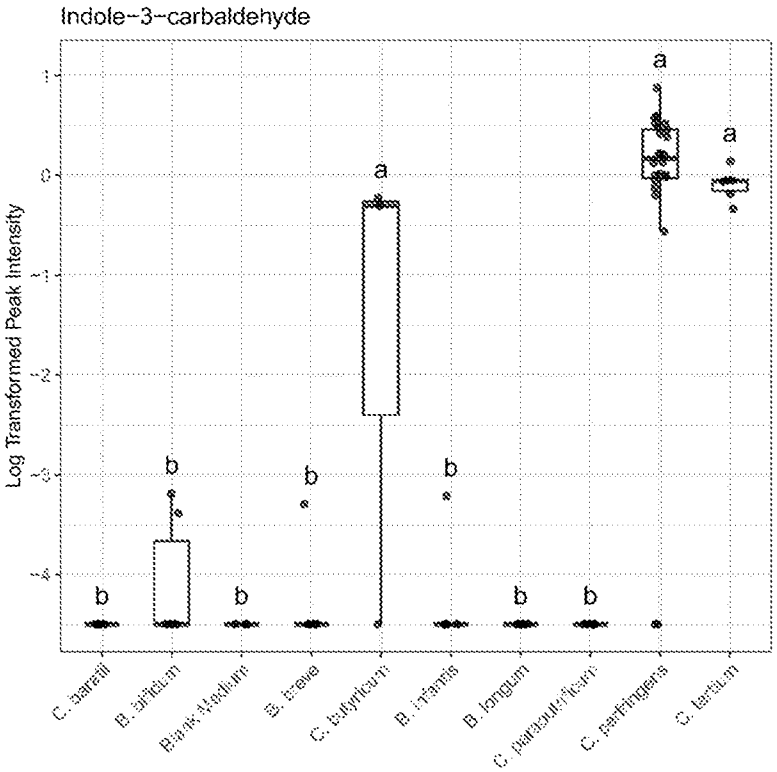


FIG. 15B

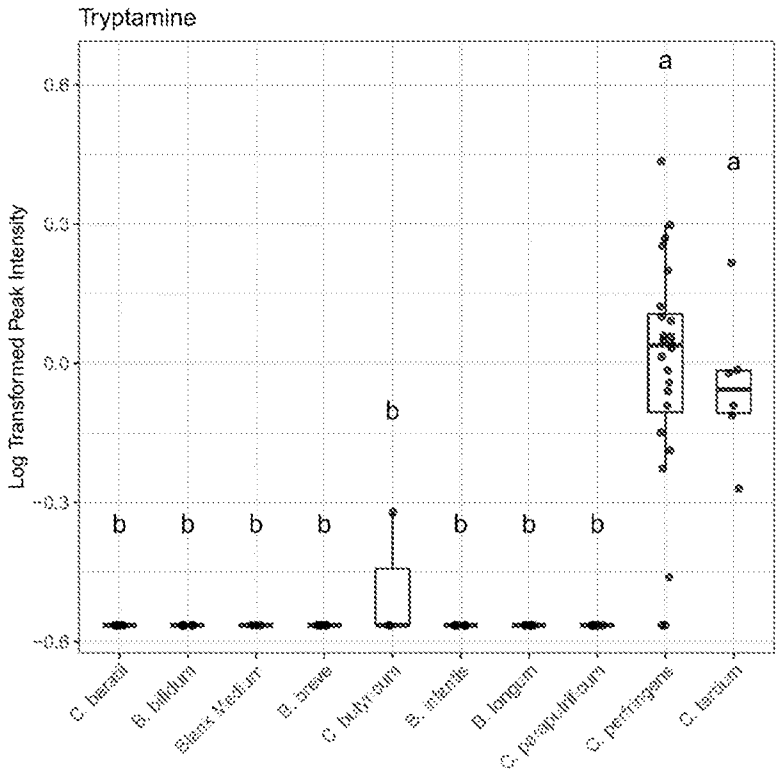


FIG. 15C

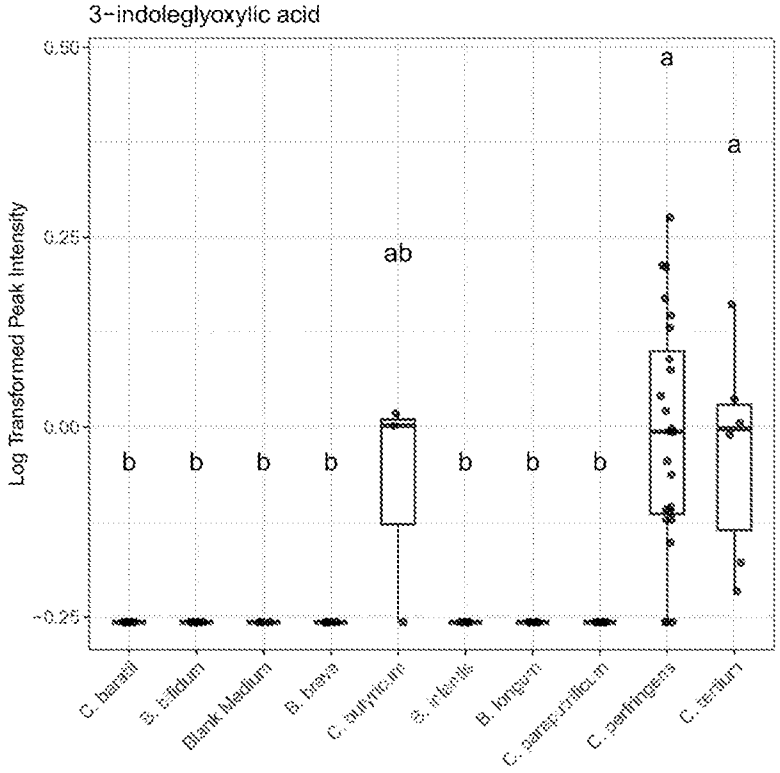


FIG. 15D

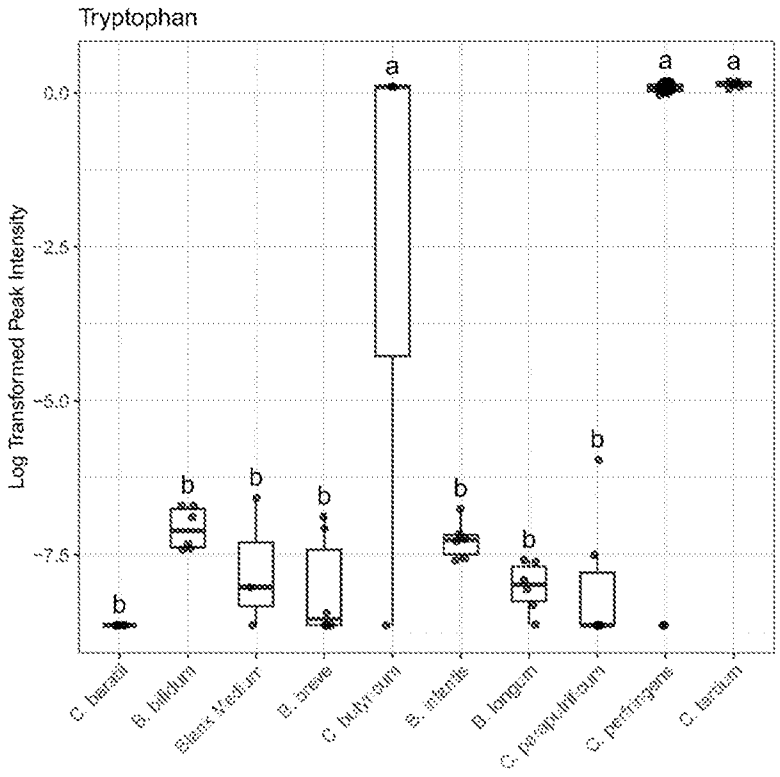


FIG. 15E

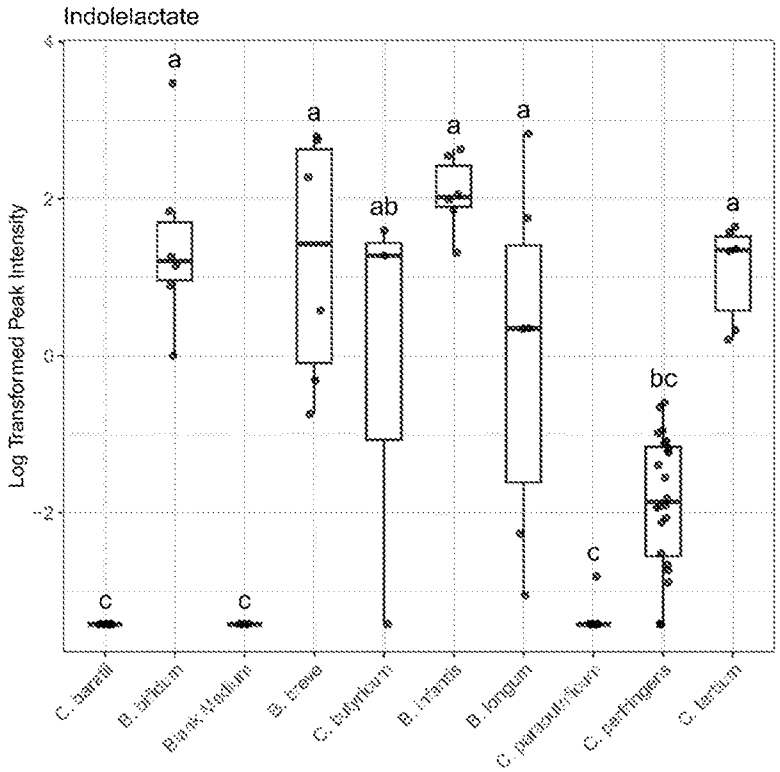


FIG. 15F

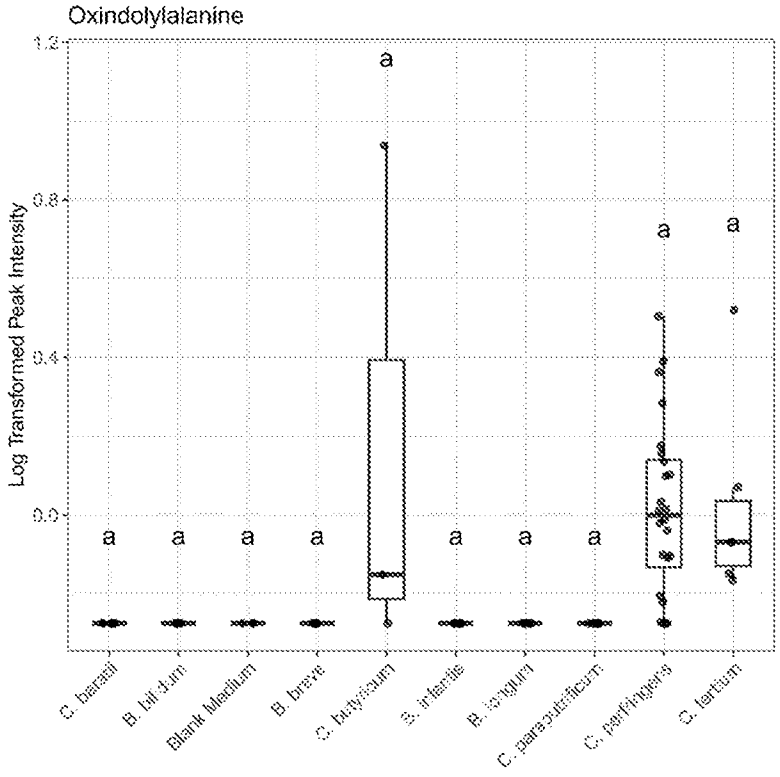


FIG. 15G

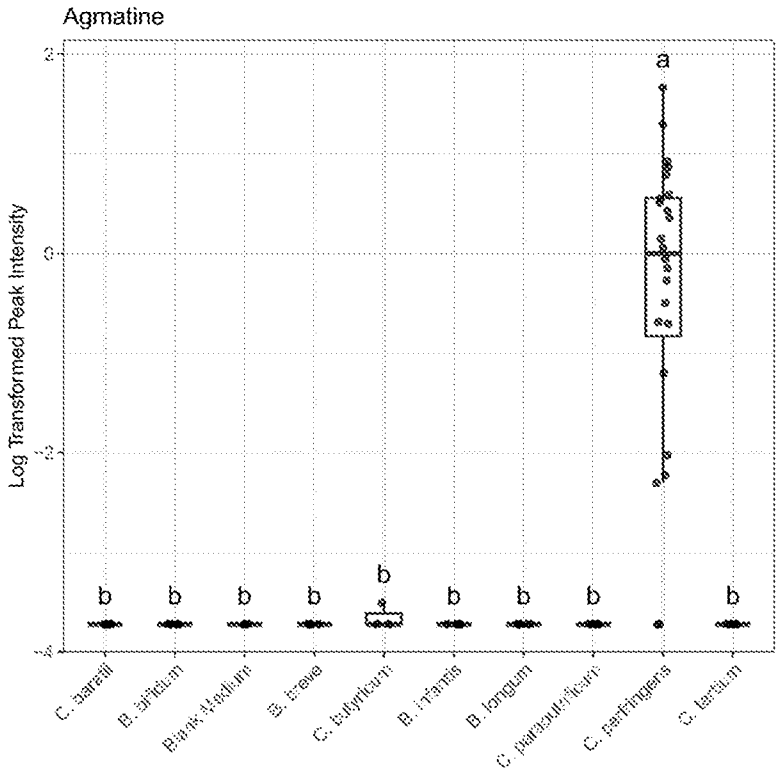


FIG. 16A

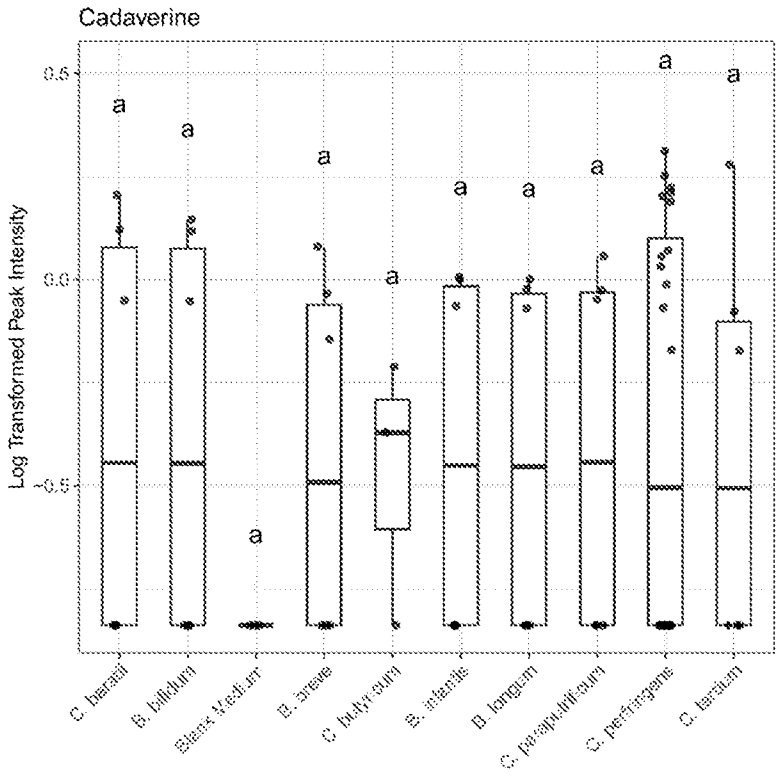


FIG. 16B

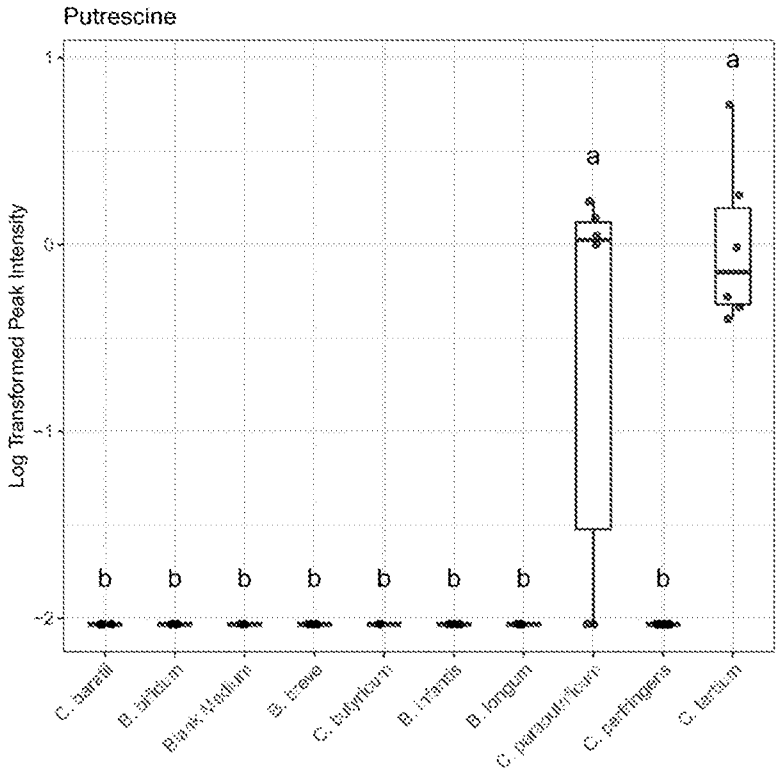


FIG. 16C

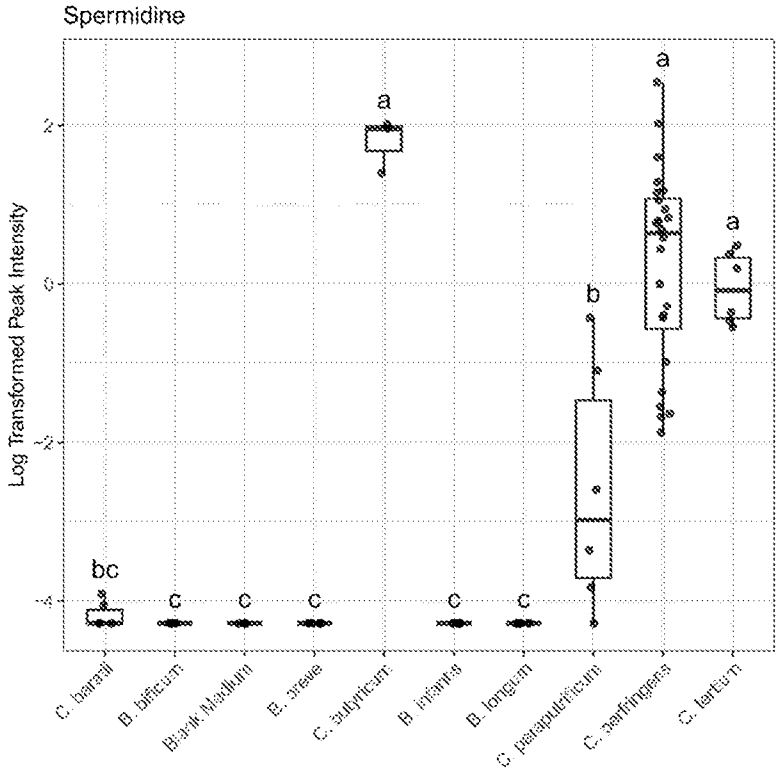


FIG. 16D

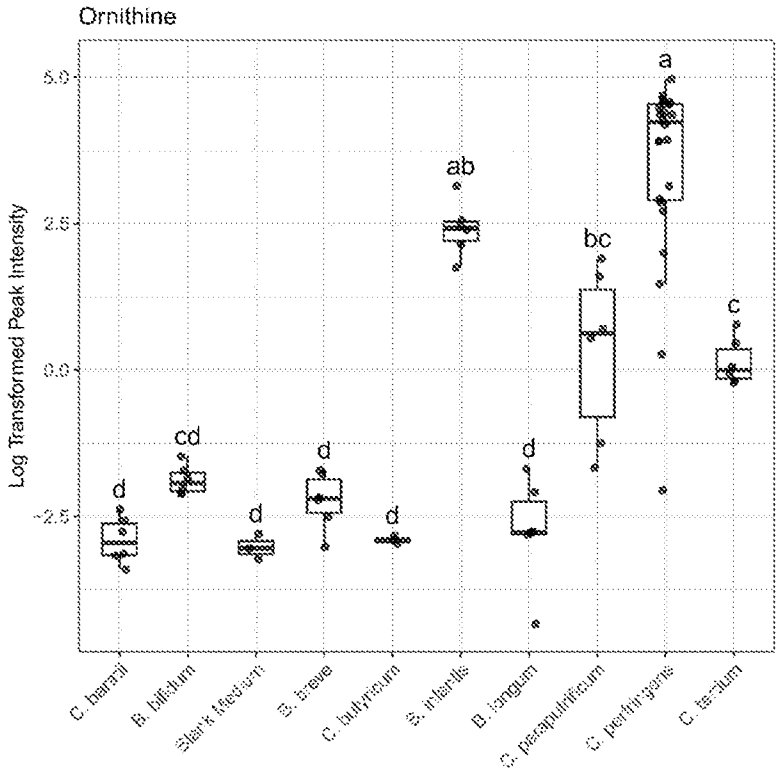


FIG. 16E

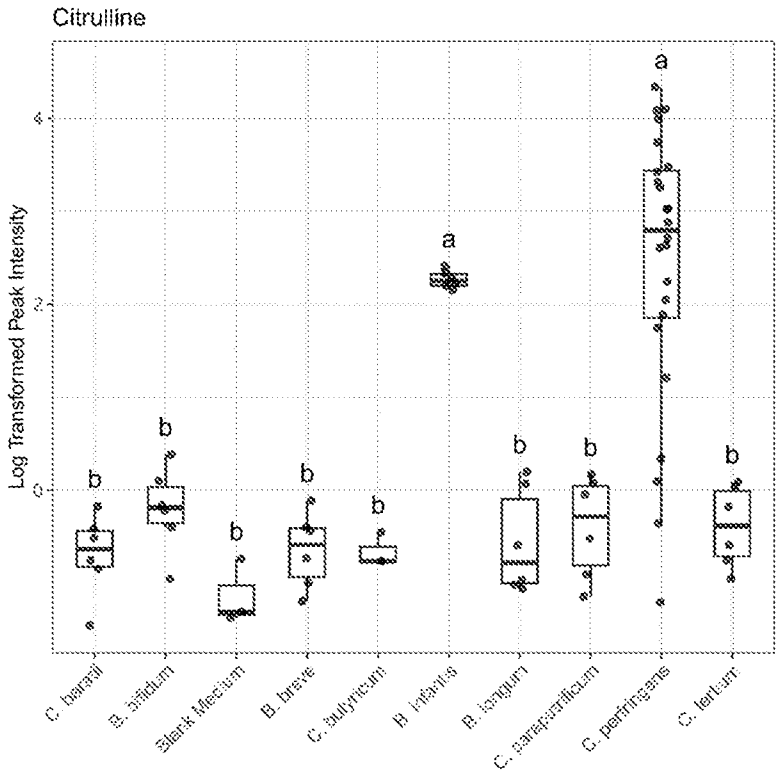


FIG. 16F

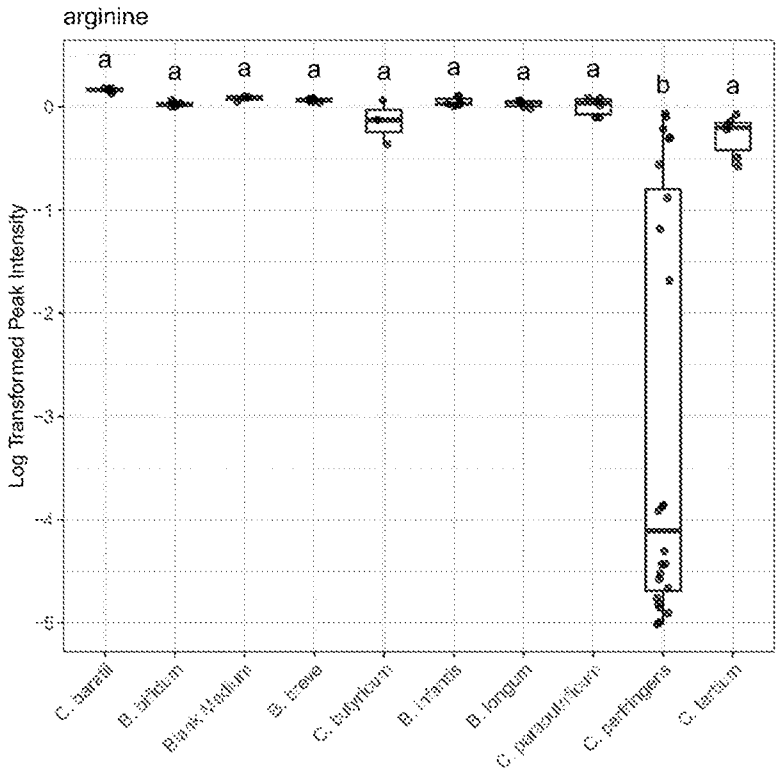


FIG. 16G

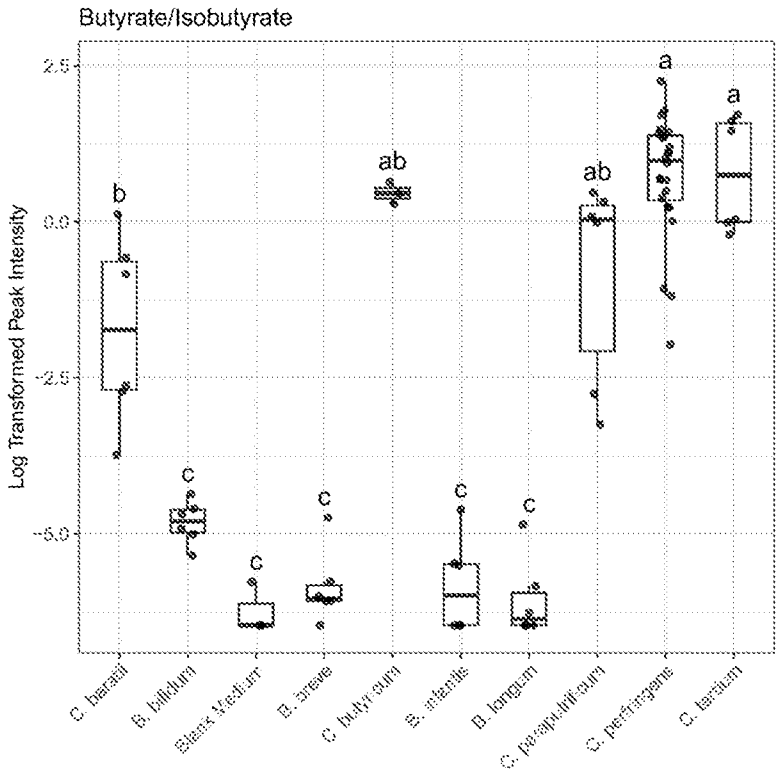


FIG. 17A

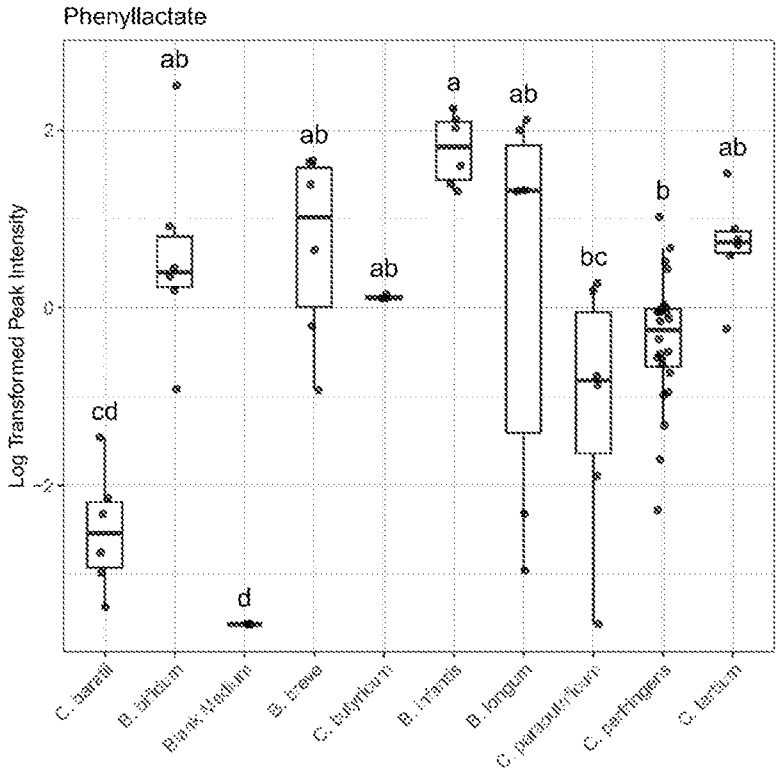


FIG. 17B

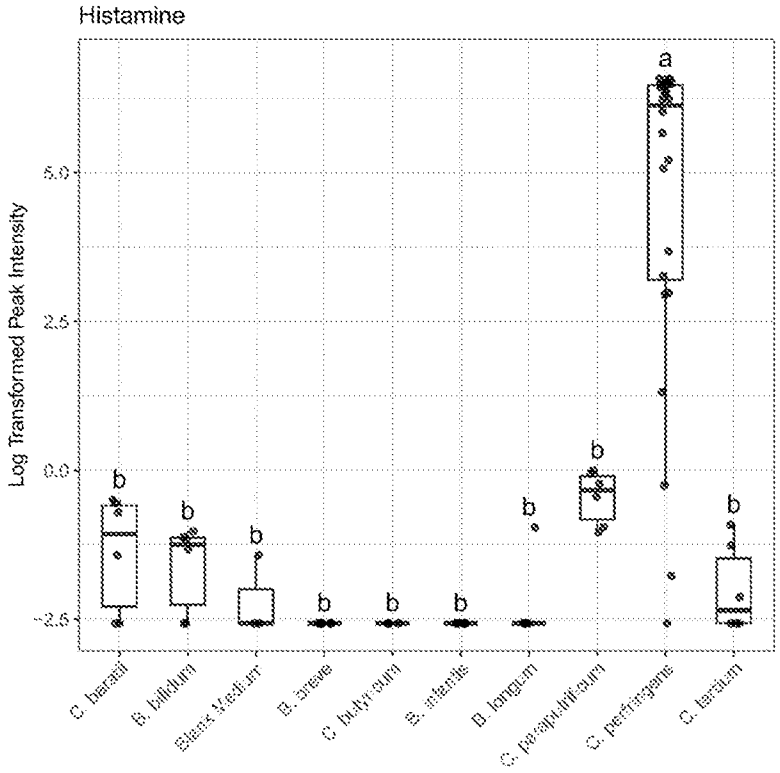


FIG. 17C

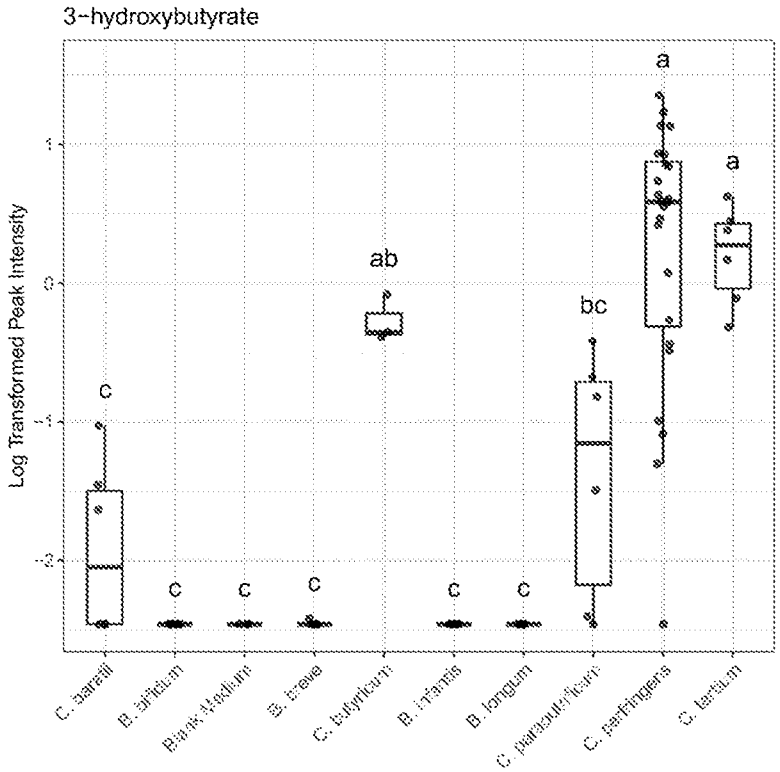


FIG. 18A

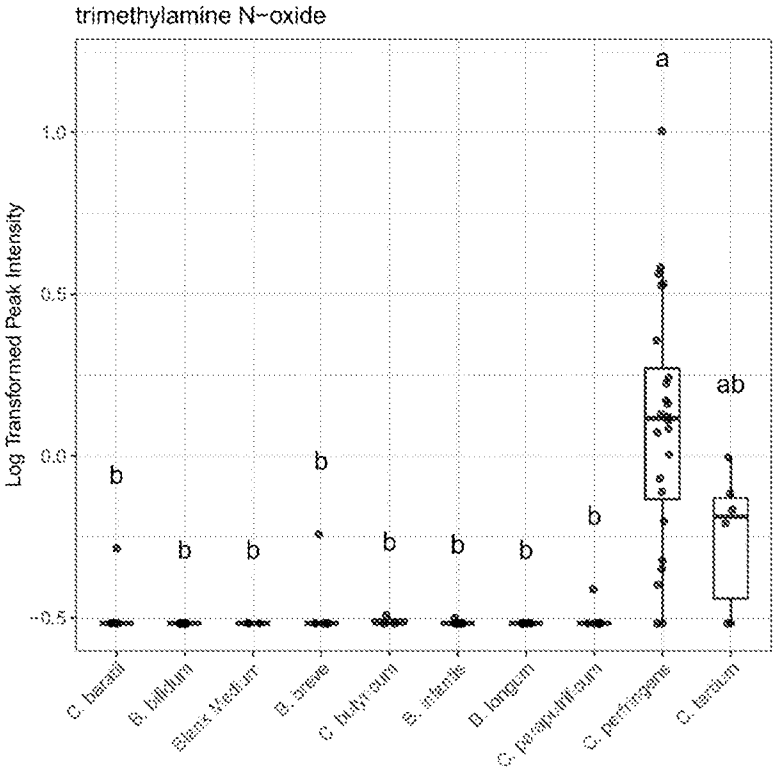

trimethylamine N~oxide
Log Transformed Peak Intensity
1.0
0.5
0.0
−0.5
a
ab
b
b
b
b
b
b
b
b
C. baratii
B. bifidum
Blank Medium
B. breve
C. butyricum
B. infantis
B. longum
C. paraputrificum
C. perfringens
C. tertium

FIG. 18B