

PROCESS

TECHNICAL FIELD

[0001] The present invention relates to processes for analysing a composition comprising therapeutic bacteria. In this application, therapeutic bacteria are defined as live bacteria which are used for the prevention, treatment or cure of a disease or condition in a mammal, preferably a human. The invention also relates to phages for use in such a process as well as a kit for conducting such a process.

BACKGROUND TO THE INVENTION

[0002] The mammalian intestine is thought to be sterile *in utero* but it is exposed to a large variety of maternal and environmental microbes immediately after birth. Thereafter, there is a dynamic period of microbial colonization and succession which provides the gut microbiota. The composition of the gut microbiota is influenced by factors such as delivery mode (e.g. via caesarean section or natural birth), environment, diet and host genotype, particularly during early life. Subsequently, the gut microbiota stabilizes and becomes adult-like. The human gut microbiota contains between about 500 and 1,000 different phylotypes belonging essentially to two major bacterial phyla, the *Bacteroidetes* and the *Firmicutes*. The successful symbiotic relationships arising from microbial colonization of the gut have yielded a wide variety of metabolic, structural, protective and other beneficial functions. The enhanced metabolic activities of the colonized gut ensure that otherwise-indigestible dietary components are degraded, releasing by-products which provide an important nutrient source for the host.

[0003] Similarly, the immunological importance of the gut microbiota is well-recognized and is exemplified in germ-free animals which have an impaired immune system that is functionally reconstituted following the introduction of commensal microbes.

[0004] Dramatic changes in the composition of the gut microbiota have been documented in human gastrointestinal disorders such as inflammatory bowel disease (IBD). For example, the levels of *Clostridium* cluster XIVa bacteria are reduced in IBD patients whilst numbers of *E. coli* bacteria are increased, suggesting a shift in the balance of symbionts and pathobionts within the gut. Interestingly, this microbial dysbiosis is also associated with imbalances in T-effector cell populations.

[0005] In recognition of the potential positive effect that certain bacterial strains may have on the mammalian gut, various bacterial strains have been proposed for use in the prevention, treatment and cure of various diseases. Examples of disclosures of the use of live bacteria to treat physiological conditions include: EP-A-1 280 541, which discloses the use of hydrogenotrophic organisms in the treatment of a range of conditions, including human irritable bowel syndrome; EP-A-1 448 995, which discloses the use of *Bacteroides thetaiotamicron* in the treatment of inflammatory diseases; EP-A-2 763 685, which discloses the use of *Roseburia hominis* as an immunoregulatory agent; WO

2017/085520, which discloses the use of *Enterococcus gallinarum* as an anti-cancer therapy; and EP-A-3206700, which discloses the use of *Bifidobacterium* in the treatment of a range of autoimmune / inflammatory conditions, including severe asthma.

[0006] Such live bacteria are used as active principals in a class of pharmaceutical agents categorised by the US FDA as Live Biotherapeutic Products ("LBP"). In guidance published by the FDA in February 2012 and updated in 2016 (Guidance for Industry: Early Clinical Trials with Live Biotherapeutic Products : Chemistry, Manufacturing, and Control Information), LBP are defined as biological products that: 1) contain live organisms, such as bacteria; 2) are applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) are not vaccines. It is further stated in the FDA's guidance document that, unlike probiotic products, LBP are subjected to the same rigorous scrutiny as pharmaceutical agents by regulatory bodies.

[0007] One challenge which the developers of LBP face is the contamination of a culture of therapeutic bacteria. If even a single cell of an organism other than the therapeutic bacterium or therapeutic bacteria is present, then these organisms can grow exponentially along with the therapeutic bacterium or therapeutic bacteria. Plainly, if such other organisms are pathogenic, then the presence of such organisms is unacceptable. Such organisms can also be problematic for other reasons, for example they may produce metabolites or express compounds whose presence in LBP is unacceptable. They may also skew the growth profile of the therapeutic bacterium or therapeutic bacteria, leading to sub-optimal production processes, which is a concern when manufacturing LBP on a commercial scale.

[0008] While those skilled in the art will be aware of approaches for assessing bacterial populations to identify the presence of unwanted organisms (such as for example bacterial strains other than therapeutic bacteria, particularly pathogenic bacteria) such approaches may not always be appropriate. For example, if an unwanted pathogenic organism is present at low levels in a biomass comprising a large population of therapeutic bacteria, it may be challenging to detect the presence of the unwanted pathogenic organism. Such a situation may arise where the therapeutic bacteria is/are effective at successfully competing with the unwanted organism for nutrients in the fermentation medium, resulting in a small or very small population of unwanted organism compared to the size of the population of the therapeutic bacteria.

[0009] While, superficially, keeping the size of the populations of unwanted organisms small or very small may appear to be attractive, in practice this is actually problematic. As explained above, to be approved as pharmaceutical products, LBP must pass through the rigorous assessments of healthcare regulators. For example, the European Pharmacopeia (Chapter 2.6.38) requires that LBP comprise less than 1000 colony forming units (CFU) of bacteria other than the therapeutic bacteria per gram of drug product. Additionally, it is also necessary to demonstrate the absence of any detectable pathogenic organisms from the *Clostridia* and *Salmonella* genera and the *Staphylococcus aureus*, *Pseudomonas*

aeruginosa and *Escherichia coli* species. In the course of regulatory processes, it is crucial that LBP developers are able to demonstrate conclusively that these requirements have been complied with.

[0010] Additionally or alternatively, even if the unwanted organisms are present at low levels in a finished product owing to their growth being suppressed by the conditions to which they are exposed during the fermentation / manufacturing of that product, when delivered to the GI tract (as the majority of LBP are), the conditions in the gut may permit the unwanted organisms to flourish and their population exponentially to increase.

[0011] Further, whether present at high or low levels, the unwanted organisms may produce metabolites or express compounds whose presence in the finished products or GI tract may be undesirable or unacceptable from a regulatory perspective. Even where the population sizes of the unwanted organisms may be low, the metabolites / products they produce may accumulate to unacceptable levels.

[0012] In *Viruses* **2015**, 7, 6675-6688, Dreher-Lesnick *et al.* disclose the development of phage lysin LysA2 for use in improved purity assays for Live Biotherapeutic Products (LBP). The authors are all from the Office of Vaccines Research and Review of the FDA. The article indicates that the process developed by the authors may prospectively be useful but the data presented do not show that it would satisfy the FDA's own requirements. For example, the authors conclude that "additional optimization is necessary to develop Lys2 and other lysins as robust reagents for use in purity assays for LBPs", and the successful use of Lys2 was only demonstrated in a colony of *L. jensenii* spiked with high levels of either *E. coli* or *S. aureus*.

[0013] Further, the process reported in that paper is conducted on purified bacterial cultures. However, as the requirements for purity assessments of LBP must be demonstrated for finished drug product, i.e. the composition to be administered to a patient, the process reported by Dreher-Lesnick would not be sufficient to demonstrate compliance with the regulatory obligations as that process has not been exemplified in connection with finished drug product.

[0014] WO 2014/153194 concerns, *inter alia*, enriching for a contaminant in a composition. Multiple alternatives and speculative assays are suggested. However, no worked example of any of these assays is provided, nor does this disclosure enable a skilled practitioner to selectively enrich for contaminants in an LBP.

[0015] There is therefore a need for an improved process for analysing pharmaceutical compositions comprising therapeutic bacteria to identify unwanted organisms, especially when those unwanted organisms are present at low levels.

SUMMARY OF THE INVENTION

[0016] The present invention provides new and improved analytical processes which allow for the fast and accurate detection of unwanted microorganisms even where the contaminants are present at low levels or where the nature of the contaminants is unknown.

[0017] In one embodiment, there is provided a process for analysing a composition comprising therapeutic bacteria, wherein the process comprises:

- (a) culturing the composition under conditions which selectively inhibit the growth of the therapeutic bacteria; and
- (b) analysing the cultured composition of step (a) to determine whether an unwanted organism was present in the composition.

[0018] The inventors have found that selective inhibition of the growth of the therapeutic bacteria advantageously enables and facilitates analysis of any unwanted organisms in the microbial composition. In an embodiment, the growth of the therapeutic bacteria only is selectively inhibited. "Selectively inhibited" preferably refers to embodiments where only the growth of the therapeutic bacteria is inhibited. In some embodiments, the growth of one or more contaminants may also be inhibited. For example, the growth of therapeutic bacteria may be inhibited at least 5 times more than the growth of one or more unwanted organisms is inhibited, such as at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, or 50 times more than the growth of one or more unwanted organisms. In an embodiment, the growth of the unwanted organism is not inhibited. In an embodiment, the growth of any unwanted organism is not inhibited.

[0019] By "selective inhibition" it is meant that the growth of the therapeutic bacteria is preferentially inhibited in culturing step (a) compared to the growth inhibition of the unwanted organism. For example, the growth inhibition of the therapeutic bacteria in step (a) may be at least two fold, at least three fold, at least four fold, at least five fold, at least ten fold, at least twenty fold, at least fifty fold higher than the growth inhibition of the unwanted organism. As a skilled person will appreciate, this is to be assessed relative to the growth rate before the selective growth conditions. Preferably, the growth rate for any unwanted organism in step (a) is at least two fold higher at least three fold higher, at least four fold higher, at least five fold higher, at least ten fold higher, at least twenty fold higher, or at least fifty fold higher than the rate at which the therapeutic bacteria grow. The reference to "unwanted organism" in this context can refer to a single unwanted organism (e.g. a single bacterial species) or it can refer to a multitude of unwanted organisms. For example, the growth inhibition of the therapeutic bacteria may be inhibited relative to all unwanted organisms in the composition or relative to only one of the unwanted organisms.

[0020] If the analysis step determines that at least one unwanted organism was present in the composition, the process may include the step of identifying one or more unwanted anaerobic or

aerobic bacteria. The bacteria may be pathogenic bacteria, such as bacteria from the *Clostridia* and *Salmonella* genera, for example bacteria from the *Clostridium difficile*, *Clostridium tetani*, or *Salmonella enterica* species, or bacteria from the *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* species, or the like, or mixtures of such unwanted organisms.

[0021] The expression "unwanted organism" refers to any organism in the composition other than the therapeutic bacteria. In some embodiments, the unwanted organism is an unwanted bacterium or unwanted bacteria. An unwanted organism may also be any bacteria that is a mammalian pathogen, such as a human pathogen. The expression "unwanted organism" is interchangeable with "contaminant" or "contaminating organism" in the context of the present specification. In an embodiment, the at least one unwanted organism is not *E. coli* or *S. aureus*. In most preferred embodiments, the unwanted organism has not been deliberately added to the composition.

[0022] Where the unwanted organism is a cellular organism, the ratio of the number of cells of the therapeutic bacteria to the number of cells of the one or more unwanted organisms in the microbial composition prior to the culturing step may be 1,000 : 1 or higher, 10,000 : 1 or higher, 100,000 : 1 or higher, 1,000,000 : 1 or higher, 10,000,000 : 1 or higher, 100,000,000 : 1 or higher, 1,000,000,000 : 1 or higher, 10,000,000,000 : 1 or higher.

Selective inhibition

[0023] Selective inhibition of the growth of the therapeutic bacteria may be achieved by selecting the pH, temperature and/or ionic strength of the culture medium. The relationship between pH and temperature and bacterial growth is well understood and methods for controlling pH and/or temperature to control bacterial growth are well known (see for example "Modelling the combined effect of temperature and pH on the rate coefficient for bacterial growth", Davey, International Journal of Food Microbiology (1994) 23 (3-4), pp 295-303). Furthermore, controlling the concentration of salts in growth media is known to inhibit the growth of certain bacteria, thus selectively inhibiting growth of sensitive bacteria (see for example "Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology", Bonnet et al., New Microbes New Infect (2020) 34: 100622).

[0024] In embodiments, step (a) in the methods of the invention is performed at a pH ≤ 7 , for example ≤ 6.5 , ≤ 6 , ≤ 5.5 , ≤ 5 , ≤ 4.5 , ≤ 4 , ≤ 3.5 , ≤ 3 , or ≤ 2.5 , for example between 2.5-6.5, 2.5-6, 2.5-5.5, 2.5-5, 2.5-4.5, 2.5-4, 2.5-3.5, 2.5-3, 3-6.5, 3-6, 3-5.5, 3-5, 3-4.5, 3-4, 3-3.5, 4-6.5, 4-6, 4-5.5, 4-5, 4-4.5, 5-6.5, 5-6, 5-5.5, or 6-6.5. In other embodiments, step (a) may be conducted at a pH of ≥ 8 , for example ≥ 8.5 , ≥ 9 , ≥ 9.5 , ≥ 10 , ≥ 10.5 , ≥ 11 , or ≥ 11.5 . For example, the pH may be between 8.5-11.5, 9-11.5, 9.5-11.5, 10-11.5, 10.5-11.5, 11-11.5, 8.5-11, 9-11, 9.5-11, 10-11, 10.5-11, 8.5-10.5, 9-10.5, 9.5-10.5, 10-10.5, 8.5-10, 9-10, 9.5-10, 8.5-9.5, 9-9.5, etc.

[0025] In addition, or alternatively, the temperature in step (a) may be selected to be $\leq 10^{\circ}\text{C}$, $\leq 9^{\circ}\text{C}$, $\leq 8^{\circ}\text{C}$, $\leq 7^{\circ}\text{C}$, $\leq 6^{\circ}\text{C}$, $\leq 5^{\circ}\text{C}$, $\leq 4^{\circ}\text{C}$, $\leq 3^{\circ}\text{C}$, $\leq 2^{\circ}\text{C}$. Where a low growth temperature is chosen it will be evident to a skilled person that the conditions need to be chosen such that the growth medium in which step (a) is conducted does not freeze. Thus, in some embodiment, the temperature may be chosen to be in a range selected from 0.5°C - 10°C , 0.5°C - 9°C , 0.5°C - 8°C , 0.5°C - 7°C , 0.5°C - 6°C , 0.5°C - 5°C , 0.5°C - 4°C , 0.5°C - 3°C , 0.5°C - 2°C , 0.5°C - 1°C , 1°C - 10°C , 1°C - 9°C , 1°C - 8°C , 1°C - 7°C , 1°C - 6°C , 1°C - 5°C , 1°C - 4°C , 1°C - 3°C , 1°C - 2°C , 2°C - 10°C , 2°C - 9°C , 2°C - 8°C , 2°C - 7°C , 2°C - 6°C , 2°C - 5°C , 2°C - 4°C , 2°C - 3°C , 3°C - 10°C , 3°C - 9°C , 3°C - 8°C , 3°C - 7°C , 3°C - 6°C , 3°C - 5°C , 3°C - 4°C , 4°C - 10°C , 4°C - 9°C , 4°C - 8°C , 4°C - 7°C , 4°C - 6°C , 4°C - 5°C , etc.

[0026] The growth of the therapeutic bacteria may also be inhibited at higher temperatures. Thus, in some embodiments, the temperature in step (a) may be selected to be $\geq 50^{\circ}\text{C}$, $\geq 55^{\circ}\text{C}$, $\geq 60^{\circ}\text{C}$, $\geq 65^{\circ}\text{C}$, $\geq 70^{\circ}\text{C}$, $\geq 75^{\circ}\text{C}$, $\geq 80^{\circ}\text{C}$, $\geq 85^{\circ}\text{C}$, or $\geq 90^{\circ}\text{C}$. It will be evident to a skilled person that it is preferable to avoid culturing beyond the boiling point. Thus, in some embodiment, the temperature may be chosen to be in a range selected from 50°C - 99°C , 55°C - 99°C , 60°C - 99°C , 65°C - 99°C , 70°C - 99°C , 75°C - 99°C , 80°C - 99°C , 85°C - 99°C , 90°C - 99°C , 50°C - 95°C , 55°C - 95°C , 60°C - 95°C , 65°C - 95°C , 70°C - 95°C , 75°C - 95°C , 80°C - 95°C , 85°C - 95°C , 90°C - 95°C , 50°C - 90°C , 55°C - 90°C , 60°C - 90°C , 65°C - 90°C , 70°C - 90°C , 75°C - 90°C , 80°C - 90°C , 85°C - 90°C , 50°C - 85°C , 55°C - 85°C , 60°C - 85°C , 65°C - 85°C , 70°C - 85°C , 75°C - 85°C , 80°C - 85°C , 50°C - 80°C , 55°C - 80°C , 60°C - 80°C , 65°C - 80°C , 70°C - 80°C , 75°C - 80°C , 50°C - 75°C , 55°C - 75°C , 60°C - 75°C , 65°C - 75°C , 70°C - 75°C , etc.

[0027] For example, enterococcal bacteria are known to survive in temperatures between 5 and 65°C , and in environments with a pH between 4.5 and 10.0, and can survive in high salt concentrations (see "The Ecology, Epidemiology, and Virulence of Enterococcus", Fisher and Phillips, Microbiology (2009) 155 (6), pp 1749-1757); when the therapeutic bacteria is enterococcal, the selective inhibition may therefore be achieved by lowering the temperature to below 5°C and/or lowering the pH to below 4.5. Likewise, several species of lactic acid bacteria, e.g. lactobacilli, are intolerant to very high pH (see for example "Diversity and Mechanisms of Alkali Tolerance in Lactobacilli", Sawatari and Yokota, Appl Environ Microbiol (2007) 73(12) pp 3909-3915); as such, when the therapeutic bacteria is a lactic acid bacteria, such as a species of lactobacilli, the selective inhibition may be achieved by raising the pH to 8.0 or higher.

[0028] The optimal growth conditions for other therapeutic bacteria are also known in the art. For example, optimum growth conditions for *Blautia hydrogenotrophica* are at a pH range of 6.0-7.0, and a temperature of 35°C - 37°C . (Bernalier *et al.* Archives of Microbiology, 1996. Volume 166(3). p. 176-83) and so a process of the invention can be practised using a pH of <6.0 or >7.0 and/or a temperature of $<35^{\circ}\text{C}$ or $>37^{\circ}\text{C}$.

Inhibitory agents

Alternatively, or in addition, selective inhibition may be achieved by addition to the culture medium of an agent or agents which selectively inhibit(s) the growth of the therapeutic bacteria. The agent may prevent the therapeutic bacteria from multiplying or may reduce multiplication, for example by at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.9%. Preferably, the agent actively kills at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9% or (most preferably) 100% of the therapeutic bacteria. Examples of such agents include bacteriophages (usually known as “phages”), lysozymes and antibiotics.

[0029] The use of phages is especially preferred because it is possible to select phages which are specific for the therapeutic bacteria and which therefore will not inhibit the growth of any unwanted organisms, even if they are also bacteria. If specific phage(s) are used, the therapeutic bacteria will be killed off and the unwanted organisms will be able to grow. The data presented in the examples demonstrate the ability of phages to rapidly inactivate high concentrations of therapeutic bacteria, thus facilitating the convenient and straightforward identification of any unwanted organisms (in those examples, bacterial organisms). The use of phages, as compared to lysozyme, is also advantageous as this avoids the additional process steps of isolating and purifying the lysozyme.

[0030] In an embodiment, the agent is not LysA2, or is not an isolated phage lytic enzyme, or is not a recombinant phage lytic enzyme. It will be understood that a phage lytic enzyme is any enzyme deriving from a bacteriophage that is capable of inducing lysis in a bacterium. In an embodiment, the agent is not an isolated phage enzyme or protein. In an embodiment, the agent is not a recombinant phage enzyme or protein. In an embodiment, the selective inhibition is not achieved by addition of an isolated and/or recombinant phage lytic enzyme to the culture medium.

[0031] Suitable phages for use in the process of the present invention include naturally occurring phages, for example any phage for which the genome is publicly available (for example via the European Nucleotide Archive (ENA) database, accessible at: <https://www.ebi.ac.uk/ena/browser/home>; or via NCBI GenBank accessible at: <https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi>). The phage may be a naturally occurring phage or non-naturally occurring phage, for example a genetically engineered or genetically modified phage. A known phage may be identified by using a microbe-phage database (for example “MVP: a microbe-phage interaction database”, Gao *et al.*, Nucleic Acids Res (2018) 46 (Database Issue): D700-D707; accessible at: <http://mvp.medgenius.info/home>; or the Virus-Host Database accessible here: <https://www.genome.jp/virushostdb/>). The specificity of a phage may be determined using any known techniques (see for example “More Is Better: Selecting for Broad Host Range Bacteriophages”, Ross *et al.*, Front Microbiol (2016) 7: 1352); for example, one may observe whether a phage is able to form plaques on a particular species or strain of host bacteria, or one may use spot testing to more

rapidly determine which bacteria are susceptible to the phage. Ross et al. also describe techniques for isolating phages from natural environments.

[0032] A phage may be genetically engineered to be more specific to a particular strain of bacteria compared to the wildtype strain, or may be engineered to be less selective compared to the wildtype strain such that it may infect any strain of a certain species. Techniques for genetically modifying and engineering phages are known in the art (see for example “Engineering Bacteriophages as Versatile Biologics”, Kilcher and Loessner, Trends in Microbiology (2019) 27(4) pp 355-367; “Approaches to optimize therapeutic bacteriophage and bacteriophage-derived products to combat bacterial infections”, Reuter and Kruger, Virus Genes (2020) 56(2) pp 136-149; “Creation of synthetic bacterial viruses”, Ando, Nihon Saikingaku Zasshi (2018) 73(4) pp 201-210; “Phage Therapy in the Era of Synthetic Biology”, Barbu et al., Cold Spring Harb Perspect Biol (2016) 8(10):a023879; “Reprogramming Bacteriophage Host Range through Structure-Guided Design of Chimeric Receptor Binding Proteins”, Dunne et al., Cell Reports (2019) 29(5) pp 1336-1350). Synthetic genome assembly and viral genome engineering can be used to create a phage with the desired properties, for example higher or lower specificity.

[0033] In preferred embodiments of the invention, a plurality of phages may be employed to achieve selective inhibition of the therapeutic bacteria, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 phages may be utilised. These phages may be specific for the same therapeutic bacteria (e.g. the same bacterial strain or species) or they may be specific for a plurality of therapeutic bacteria (for example a plurality of bacterial species). The latter embodiment is particularly useful where the invention is used for testing compositions comprising bacterial consortia. The invention also provides compositions comprising such phages optionally in combination with a stabiliser, preservative and / or additive.

[0034] Examples of phages which may be used with the invention include: Φ CrAss001 (specific to *Bacteroides* bacteria), B124-14 (specific to *Bacteroides* bacteria), B40-8 (specific to *Bacteroides* bacteria), AUEF3 (specific to *Enterococcus* bacteria), BC611 (specific to *Enterococcus* bacteria), Ec-ZZ2 (specific to *Enterococcus* bacteria), A2 (specific to *Lactobacillus* bacteria), 521B (specific to *Lactobacillus* bacteria), c2 (specific to *Lactobacillus* bacteria), or ϕ iCDHM11 (specific to *Clostridium* bacteria).

[0035] The phage may be specific for a Gram positive bacterium, for example bacteria of the genus *Enterococcus*. The phage may have a DNA genome. The phage may be specific for enterococcal bacteria, and optionally cannot infect non-enterococcal bacteria. The phage may selectively inhibit the growth of only enterococcal bacteria. Enterococcus-specific phages are of significant value to the process of the present invention as, to the inventors' knowledge, no phages of sufficient specificity and potency to be used in the process of the present invention had been known previously, nor had any Enterococcus-specific antibiotics been available.

[0036] Preferably, the phage have high specificity and high selectivity for a therapeutic bacterium. In an embodiment, the phage(s) inhibits the growth of no more than 10 different species of bacteria, such as no more than 9, 8, 7, 6, 5, 4, 3, 2, or 1 species of bacteria, preferably wherein each of the different species is from the same genus. In an embodiment, the phage(s) inhibits the growth of only one species of bacterium. In an embodiment, the phage(s) inhibits the growth of no more than 10 different strains of bacteria, such as no more than 9, 8, 7, 6, 5, 4, 3, 2, or 1 strains of bacteria, preferably wherein each of the different strains is from the same species. In an embodiment, the phage(s) inhibits the growth of only one strain of bacterium. In an embodiment, the phage(s) has known specificity and/or known selectivity.

[0037] In an embodiment, the phage(s) selectively inhibits the growth of the therapeutic bacteria in the composition. In an embodiment, the phage(s) selectively inhibits the growth of the therapeutic bacteria, and any bacteria of the same species as the therapeutic bacteria. In an embodiment, the phage(s) inhibits the growth of only the therapeutic bacteria in the composition. In an embodiment, the phage(s) does not inhibit the growth of any unwanted organisms in the composition.

[0038] When the therapeutic bacteria are enterococcal bacteria, particularly suitable phages include NCIMB 43666 phage and NCIMB 43667 phage (both deposited at NCIMB Ltd. Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA Scotland by 4D Pharma Research Ltd. of Life Sciences and Innovation Building, Cornhill Road, Aberdeen, AB25 2ZS), both of which are specific for enterococcal bacteria, particularly *Enterococcus gallinarum*, such as *Enterococcus gallinarum* strain NCIMB 42488 (also deposited at NCIMB Ltd. Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA Scotland by 4D Pharma Research Ltd. of Life Sciences and Innovation Building, Cornhill Road, Aberdeen, AB25 2ZS). It has been found that these phages are highly specific for *Enterococcus gallinarum* (in particular *Enterococcus gallinarum* strain NCIMB 42488) and do not affect other, unwanted microorganisms that may be present in the medium. These phages are therefore suitable agents for specifically inhibiting the growth of *Enterococcus gallinarum*, such as *Enterococcus gallinarum* strain NCIMB 42488.

[0039] In an embodiment, the phage selectively inhibits the growth of only bacteria of the species *Enterococcus gallinarum*. In an embodiment, the phage selectively inhibits the growth of *Enterococcus gallinarum* strain NCIMB 42488 bacteria. In an embodiment, the phage inhibits the growth of only *Enterococcus gallinarum* bacteria. In an embodiment, the phage inhibits the growth of only *Enterococcus gallinarum* strain NCIMB 42488 bacteria.

[0040] Thus, the NCIMB 43667 phage is preferred for use in the present invention, preferably when the therapeutic bacteria comprise or consist of bacteria of the genus *Enterococcus*. Preferably, this phage is used when the therapeutic bacteria comprise or consist of bacteria of the species *Enterococcus gallinarum*, most preferably *Enterococcus gallinarum* strain NCIMB 42488. The nucleic acid sequence for the genome of NCIMB 43667 is set out in SEQ ID NO: 1. Each of SEQ ID NOs: 3 to 68

represent a separate node of the genome of NCIMB 43667, wherein each node is a scaffold or contig assembled during sequencing of the phage genome. At least any nodes over 1000 base pairs long are likely to represent a real portion of the phage genome. In an embodiment, therefore, the nucleic acid sequence of the genome of NCIMB 43667 comprises at least one, at least two, at least three, at least 4, at least 5 or all of SEQ ID NOs: 3 to 8, in any order. NCIMB 43667 and any variant thereof which has the same or similar activity in the assays described below is also a part of the present invention. Further details of this phage are disclosed in the Examples below. A variant of the NCIMB 43667 phage may have a DNA genome that is at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% identical to SEQ ID NO: 1. A variant of the NCIMB 43667 phage may have a DNA genome that is at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% identical to SEQ ID NOs: 3 to 8 or 3 to 68. The process of the invention can also be practised using a phage which is functionally equivalent to the NCIMB 43667 phage (such a functionally equivalent phage may have a DNA genome with sequence identity to SEQ ID NO: 1 as defined in the preceding sentence). "Functionally equivalent" means that the phage can inhibit the growth of *Enterococcus gallinarum* strain NCIMB 42488 to the same extent, or at least 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.9% compared to NCIMB 43667 phage. The growth inhibition can be determined easily in a pilot experiment in which growth inhibition of the NCIMB 43667 and of the phage of interest are measured in parallel and compared. Growth inhibition may be measured by plating a mixture of the phage and *Enterococcus gallinarum* strain NCIMB 42488 on agar and inspecting for plaque formation in the resulting bacterial lawn (for example, 100 µL of pre-determined phage dilution combined with 500 µL of *Enterococcus gallinarum* strain NCIMB 42488 culture, added to 3mL top agar at 42°C, then distributed over Casein peptone Soybean peptone (CASO) agar and incubated for 37°C for between 16 and 48 hours, prior to plaque formation inspection). The number of plaques or plaque forming units may then, for example, be used to indicate growth inhibition.

[0041] The NCIMB 43666 phage is also preferred for use in the present invention. This phage is particularly useful when the therapeutic bacteria comprise or consist of bacteria of the genus *Enterococcus*. Preferably, this phage is used when the therapeutic bacteria comprise or consist of bacteria of the species *Enterococcus gallinarum*, most preferably *Enterococcus gallinarum* strain NCIMB 42488. The nucleic acid sequence for the genome of NCIMB 43666 is set out in SEQ ID NO: 2. Each of SEQ ID NOs: 69 to 2626 represent a separate node of the genome, wherein each node is a scaffold or contig assembled during sequencing of the phage genome. At least any nodes over 1000 base pairs long is likely to represent a real portion of the phage genome. In an embodiment, therefore,

the nucleic acid sequence of the genome of NCIMB 43667 comprises at least one, at least two, at least three, at least 4, at least 5 or all of SEQ ID NOs: 69 to 74, in any order. NCIMB 43666 and any variant thereof which has the same or similar activity in the assays described below is also a part of the present invention. Further details of this phage are disclosed in the Examples below. A variant of the NCIMB 43666 phage according to the invention may have a DNA genome that is at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% identical to SEQ ID NO: 2. A variant of the NCIMB 43666 phage according to the invention may have a DNA genome that is at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% identical to at least one, at least two, at least three, at least 4, or at least 5 or all of SEQ ID NOs: 69 to 74, or one, some or all of 69 to 2626. The process of the invention can also be practised using a phage which is functionally equivalent to the NCIMB 43666 phage (such a functionally equivalent phage may have a DNA genome with sequence identity SEQ ID NO: 2 as defined in the preceding sentence). "Functionally equivalent" means that the phage can inhibit the growth of *Enterococcus gallinarum* strain NCIMB 42488 to the same extent, or at least 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.9% compared to NCIMB 43666. The growth inhibition can be determined easily in a pilot experiment in which growth inhibition of the NCIMB 43666 and of the phage of interest are measured in parallel and compared. Growth inhibition may be measured by plating a mixture of the phage and *Enterococcus gallinarum* strain NCIMB 42488 on agar and inspecting for plaque formation in the resulting bacterial lawn (for example, 100 µL of pre-determined phage dilution combined with 500 µL of *Enterococcus gallinarum* strain NCIMB 4248 culture, added to 3mL top agar at 42°C, then distributed over Casein peptone Soybean peptone (CASO) agar and incubated for 37°C for between 16 and 48 hours, prior to plaque formation inspection). The number of plaques or plaque forming units may then, for example, be used to indicate growth inhibition.

[0042] References to a percentage sequence identity between two nucleotide sequences refers to the percentage of nucleotides that are the same in comparing the two sequences when aligned. This alignment and percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987) Supplement 30, in "Review of Common Sequence Alignment Methods: Clues to Enhance Reliability", *Current Genomics* (Lambert *et al.*, 2003), in "Multiple sequence alignment modeling: methods and applications", *Briefings in Bioinformatics* (Chatzou *et al.*, 2016), or in *Statistics for Bioinformatics: Methods for Multiple Sequence Alignment* (Thompson, 2017, published by ISTE Press - Elsevier). The sequence alignment may be local or global, and is preferably

global. A preferred local alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM62 matrix. The Smith-Waterman homology search algorithm is disclosed in Smith & Waterman (1981) *Adv. Appl. Math.* 2: 482-489. Local alignment may also be achieved by the Smith-Waterman homology search algorithm using a BLOSUM50 matrix. A preferred global alignment is determined by the Needleman-Wunsch algorithm, disclosed in Needleman & Wunsch (1970) *Journal of Molecular Biology*, 48(3):443-453.

[0043] The combination of NCIMB 43667 and NCIMB 43666 phages (or variants and/or functional equivalents of either, as defined above) is particularly preferred for use in the present invention, especially when the therapeutic bacteria comprise or consist of bacteria of the genus *Enterococcus*, as the inventors have seen particularly good results with the combination of these phages. Preferably, this combination of phages is used when the therapeutic bacteria comprise or consist of bacteria of the species *Enterococcus gallinarum*, most preferably *Enterococcus gallinarum* strain NCIMB 42488.

[0044] In embodiments of the invention in which the unwanted organism is a bacterium, the use of a phage as the agent is especially preferred. The data presented in the examples demonstrate the ability of phage to rapidly inactivate high concentrations of therapeutic bacteria, thus facilitating the convenient and straightforward identification of any unwanted organisms (in those examples, bacterial organisms). The use of phages, as compared to lysozyme, is also advantageous as this avoids the additional process steps of isolating and purifying the lysozyme.

[0045] Alternatively, or in addition, a process of the invention can be practised using a lysozyme. Lysozyme, also known as a muramidase or an N-acetylmuramidoglycanhydrolase, is an antimicrobial enzyme produced by animals that forms part of the innate immune system. Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of Gram-positive bacterial cell walls. This hydrolysis in turn compromises the integrity of bacterial cell walls causing lysis of the bacteria.

[0046] Specific lysozymes which may be employed in the present invention may be selected from any of the known lysozymes and are of particular use where the therapeutic bacteria are Gram-positive bacteria and the one or more unwanted organisms are not Gram-positive bacteria. The lysozyme may be naturally occurring or non-naturally occurring. For example, the lysozyme may have been engineered, for example to increase specificity for the therapeutic bacteria. The lysozyme may be a c-type lysozyme (chicken-type or conventional-type), a g-type lysozyme (goose-type), or i-type lysozyme (invertebrate type). Multiple lysozymes may be used, for example a combination of hen lysozyme and human lysozyme may be used. A non-naturally occurring lysozyme may also be used, for example a lysozyme which has been artificially mutated, a recombinant lysozyme, or a lysozyme fusion protein.

Antibiotics

[0047] The invention can also be practised using antibiotics. These embodiments are particularly suitable where the unwanted organism is not bacterial, but is a virus, for example.

[0048] Antibiotics are commonly classified based on their mechanism of action, chemical structure, or spectrum of activity. Most target bacterial functions or growth processes. An antibiotic may be bactericidal or bacteriostatic. Those that target the bacterial cell wall (penicillins and cephalosporins) or the cell membrane (polymyxins) or interfere with essential bacterial enzymes (rifamycins, lipiarmycins, quinolones and sulfonamides) have bactericidal activities. Protein synthesis inhibitors (macrolides, lincosamides and tetracyclines) are usually bacteriostatic (with the exception of bactericidal aminoglycosides). Further categorization is based on their target specificity. "Narrow-spectrum" antibiotics target specific types of bacteria, such as Gram-negative or Gram-positive, whereas broad-spectrum antibiotics affect a wide range of bacteria.

[0049] Specific antibiotics that may be used in the present invention can readily be selected based on the therapeutic bacteria which need(s) to be inhibited and the one or more unwanted organisms which are to be allowed to grow. The antibiotic may be bactericidal or bacteriostatic. The antibiotic may be a naturally occurring antibiotic, a synthetic antibiotic, or a semisynthetic antibiotic.

[0050] The antibiotic is preferably a narrow spectrum antibiotic to ensure that unwanted organisms are not affected. Suitable examples of narrow-spectrum antibiotics include penicillin G, which is mainly effective against Gram-positive bacteria, vancomycin and macrolides, which are also mainly effective against Gram-positive bacteria, particularly staphylococcal bacteria, temocillin, which is mainly effective against Gram-negative bacteria. An antibiotic specific to Gram-negative bacteria is preferably used when the therapeutic bacteria are Gram-negative and the one or more unwanted organisms are not Gram-negative bacteria. An antibiotic specific to Gram-positive bacteria is preferably used when the therapeutic bacteria are Gram-positive and the one or more unwanted organisms are not Gram-positive bacteria. For example, when the therapeutic bacteria are enterococcal bacteria, a narrow-spectrum antibiotic specific to Gram-positive bacteria is preferably used.

[0051] Alternatively, in an embodiment, the growth of the therapeutic bacteria is selectively inhibited by addition to the culture medium of an agent which inhibits the growth of the therapeutic bacteria, with the proviso that the agent is not an antibiotic. In an embodiment, the agent is not vancomycin. In an embodiment, the agent is not sodium phosphate or glycerophosphate.

[0052] In an embodiment, the selective inhibition is not achieved by addition of an antibiotic.

Combinations

[0053] The invention can also be practised using a combination of the inhibitory methods discussed in the preceding paragraphs. The same or different inhibitory methods may be employed. For

example, a process according to the invention may involve a step of growing the microbial composition at a low pH in the presence of a lysozyme. Alternatively, a process according to the invention may involve a step of growing the microbial composition in the presence of two types of phage.

The composition

[0054] The microbial composition according to the invention is most preferably a LBP. In some embodiments, it may be a vaccine.

[0055] In preferred embodiments, the microbial composition is pharmaceutically acceptable before step (a) of the process of the invention. Most preferably, no contaminating bacteria and/or viruses are added to the microbial composition.

[0056] In embodiments of the invention, the microbial composition is a finished drug product composition or unit dose (e.g. a single tablet or capsule). For example, the microbial composition may comprise excipients in addition to the therapeutic bacteria, such as lyoprotectants, preservatives, antioxidants, stabilisers, prebiotic compounds, pharmaceutically acceptable carriers or diluents, binders, lubricants, suspending agents, coating agents, solubilising agents, or the like. Additionally or alternatively, the microbial composition may comprise 1×10^{10} CFU or less, 10^{11} or less of therapeutic bacteria. As demonstrated in the examples, the process of the present invention is sufficiently sensitive to enable the presence or absence of unwanted bacteria to be determined in a single dosage form. Further, the processes of the invention do not require the separation of the therapeutic bacteria from the excipients prior to the commencement of the assessment of microbial purity. The single dosage form may be an enteric formulation, i.e. a gastro-resistant formulation (for example, resistant to gastric pH) that is suitable for delivery of the composition to the intestine by oral administration. The single dosage form may comprise an enteric coating.

[0057] The microbial composition may be a sample taken from a bulk. The sample may then be analysed according to the invention. If the results indicate the absence of unwanted organisms, the bulk may then be processed further, for example to prepare a unit dose of a medicament.

[0058] When the microbial composition is a vaccine, the therapeutic bacteria may be pathogenic bacteria, or optionally non-virulent strains of pathogenic bacteria. The therapeutic bacteria in the vaccine microbial composition may therefore also be alive in the finished drug product composition or unit dose. In an embodiment, the vaccine microbial composition does not comprise attenuated therapeutic bacteria. Alternatively or in addition, the therapeutic bacteria may be genetically engineered non-pathogenic bacteria that comprise one or more antigens from one or more pathogenic bacteria, such that inoculation with the live non-pathogenic bacteria immunises the patient against the one or more pathogenic bacteria. In an embodiment, the genetically engineered non-pathogenic bacteria express one or more antigens from one or more pathogenic bacteria. In an

embodiment, the genetically engineered non-pathogenic bacteria comprise one or more plasmids that comprise nucleic acids encoding one or more antigens from one or more pathogenic bacteria.

[0059] In an embodiment, the process of the invention comprises the step of manufacturing a microbial composition, wherein the microbial composition is a finished drug product composition suitable for administration to a patient, optionally a human patient, prior to the steps of culturing a microbial composition comprising therapeutic bacteria under conditions which selectively inhibit the growth of the therapeutic bacterium or therapeutic bacteria, and analysing the cultured microbial composition to determine whether any unwanted organism was present in the microbial composition.

[0060] The composition may comprise more than one species or strain of therapeutic bacteria. If the bacteria comprise multiple strains, they may be from different phyla, class, order, family, genera, or species, or may be different strains from the same species. For example, the multiple therapeutic bacterial strains may be: of the same phyla but different class; of the same class but different order; of the same order but different family; of the same family but different genera; of the same genera but different species; or of the same species but different strains. Microbial compositions comprising combinations of these categories are also encompassed by the present invention, for example the therapeutic bacteria may comprise two or more therapeutic bacterial strains of the same genera but different species and two or more therapeutic bacterial strains of the same species but different strains. The microbial composition may comprise multiple different therapeutic bacterial strains of the phylum *Bacteroidetes*, of the phylum *Firmicutes*, or both. The microbial composition may comprise multiple species of therapeutic bacteria of the genus *Enterococcus*. The microbial composition may comprise multiple strains of therapeutic bacteria of the species *Enterococcus gallinarum*. In an embodiment, the microbial composition does not comprise *L. jensenii*. In an embodiment, the therapeutic bacteria does not comprise *L. jensenii*.

[0061] The microbial composition may comprise a single therapeutic bacterial strain. Alternatively, the microbial composition may comprise a consortium of therapeutic bacterial strains, for example it may comprise a plurality of therapeutic bacterial strains. For example, in some embodiments, the microbial composition may comprise more than one strain from within the same species (e.g. more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40 or 45 strains) and, optionally, does not contain therapeutic bacteria from any other species. In some embodiments, the therapeutic bacteria may comprise fewer than 50 strains from within the same species (e.g. fewer than 45, 40, 35, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4 or 3 strains) and, optionally, does not contain therapeutic bacteria from any other species. In some embodiments, the microbial composition comprises 1-40, 1-30, 1-20, 1-19, 1-18, 1-17, 1-15, 1-10, 1-9, 1-8, 1-7, 1-6, 1-5, 1-4, 1-3, 1-2, 2-50, 2-40, 2-30, 2-20, 2-15, 2-10, 2-5, 6-30, 6-15, 16-25 or 31-50 strains from within the same species and, optionally, does not contain therapeutic bacteria from any other species.

[0062] In some embodiments, the microbial composition comprise therapeutic bacterial strains from more than one species (for example more than one species from within the same genus), e.g. more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35 or 40 species and, optionally, does not contain therapeutic bacterial strains from any other genus. In some embodiments, the microbial composition comprises therapeutic bacterial strains from fewer than 50 species (for example fewer than 50 species from within the same genus), e.g. fewer than 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 12, 10, 8, 7, 6, 5, 4 or 3 species) and, optionally, does not contain therapeutic bacteria from any other genus. In some embodiments, the therapeutic bacteria comprises strains from 1-50, 1-40, 1-30, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 1-10, 1-9, 1-8, 1-7, 1-6, 1-5, 1-4, 1-3, 1-2, 2-50, 2-40, 2-30, 2-20, 2-15, 2-14, 2-13, 2-12, 2-11, 2-10, 2-5, 6-30, 6-15, 16-25, or 31-50 species from within the same genus and, optionally, does not contain therapeutic bacteria from any other genus. The microbial composition may comprise any combination of the foregoing. In some embodiments, the microbial composition comprises a consortium of therapeutic bacterial strains. In some embodiments, the consortium of therapeutic bacterial strains comprises two or more therapeutic bacterial strains obtained from a faeces sample of a single organism, e.g. a human. In some embodiments, the consortium of therapeutic bacterial strains is not found together in nature. For example, in some embodiments, the consortium of therapeutic bacterial strains comprises therapeutic bacterial strains obtained from faeces samples of at least two different organisms. In some embodiments, the two different organisms are from the same species, e.g. two different humans. In some embodiments, the two different organisms are an infant human and an adult human. In some embodiments, the two different organisms are a human and a non-human mammal.

[0063] The therapeutic bacteria may comprise or consist of anaerobic bacteria, obligate anaerobic bacteria, facultative anaerobic bacteria and / or microaerophilic bacteria.

[0064] The therapeutic bacteria may comprise or consist of bacteria from the following genera: *Enterococcus* (e.g. *Enterococcus gallinarum*, *Enterococcus caselliflavus*, *Enterococcus faecalis* or *Enterococcus faecium*), *Blautia* (e.g. *Blautia hydrogenotrophica*, *Blautia stercoris*, *Blautia wexlerae* or *Blautia producta*), *Bacteroides* (e.g. *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides vulgatus*, *Bacteroides dorei* or *Bacteroides copricola*), *Faecalibacterium* (e.g. *Faecalibacterium prausnitzii*), *Bariatricus* (e.g. *Bariatricus massiliensis*), *Bifidobacterium* (e.g. *Bifidobacterium breve*, *Bifidobacterium adolescentis* or *Bifidobacterium longum*), *Roseburia* (e.g. *Roseburia hominis*, *Roseburia intestinalis* or *Roseburia inulinivorans*), *Flavonifractor* (e.g. *Flavonifractor plautii*), *Anaerotruncus* (e.g. *Anaerotruncus colihominis*), *Parabacteroides* (e.g. *Parabacteroides distasonis*, *Parabacteroides goldsteinii*, *Parabacteroides merdae* or *Parabacteroides johnsonii*), *Erysipelatoclostridium* (e.g. *Erysipelatoclostridium ramosum*), *Megasphaera* (e.g. *Megasphaera massiliensis*), *Pediococcus* (e.g. *Pediococcus acidilacticii*), *Eubacterium* (e.g. *Eubacterium contortum*, *Eubacterium fissicatena*,

Eubacterium eligens, *Eubacterium hadrum*, *Eubacterium hallii* or *Eubacterium rectale*), *Ruminococcus* (e.g. *Ruminococcus torques*, *Ruminococcus gnavus* or *Ruminococcus bromii*), *Pseudoflavonifractor* (e.g. *Pseudoflavonifractor capillosus*), *Clostridium* (e.g. *Clostridium nexile*, *Clostridium hylemonae*, *Clostridium butyricum*, *Clostridium tertium*, *Clostridium disporicum*, *Clostridium bifermentans*, *Clostridium inocuum*, *Clostridium mayombeii*, *Clostridium bolteae*, *Clostridium bartletti*, *Clostridium symbiosum* or *Clostridium orbiscindens*), *Coprococcus* (e.g. *Coprococcus comes* or *Coprococcus catus*), *Acetivibrio* (e.g. *Acetivibrio ethanolignens*), *Dorea* (e.g. *Dorea longicatena*) or any genera of the family *Lachnospiraceae*.

[0065] In embodiments of the invention, the microbial composition does not comprise organisms from the *Lactobacillus* genus and / or the *Lactobacillaceae* family.

[0066] The microbial composition may have any therapeutic use, such as use in the treatment or prevention of cancer, gastrointestinal disease (such as inflammatory bowel disease, inflammatory bowel syndrome, Crohn's disease), diabetes, periodontitis, or metabolic diseases (such as metabolic syndrome). In an example, the therapeutic bacteria have therapeutic use in the treatment or prevention of cancer. The therapeutic bacteria may be pathogenic or non-pathogenic to mammals, e.g. humans. The therapeutic bacteria may comprise naturally occurring and / or artificial bacteria, such as genetically modified therapeutic bacteria.

Detection

[0067] Any unwanted organisms in the microbial composition are preferably live at the beginning of the culture step.

[0068] Examples of unwanted organisms which may be present in the microbial composition include bacteria. For example, the unwanted organism could be an anaerobic bacterium which may be pathogenic (e.g. a pathogenic bacterium from the *Clostridia* or *Salmonella* genera or a *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Escherichia coli* species), e.g. an anaerobic bacterium which is pathogenic to mammals, such as humans.

[0069] Identification of any unwanted organisms may be achieved using techniques or apparatuses well known to those skilled in the art. For example, organisms may be identified using one or more of the following techniques: microorganism phenotype analysis (such as identifying morphology, Gram staining, and / or sensitivity to antimicrobials, see for example Chapter 3: "Classification", of Medical Microbiology, 4th edition, 1996, edited by Samuel Baron, or "Methods for the detection and identification of pathogenic bacteria: past, present, and future", Varadi et al., Chem Soc Rev (2017) 46(16) pp 4818-4832); mass spectrometry (such as MALDI-TOF-MS; see for example "Rapid and Robust MALDI-TOF MS Techniques for Microbial Identification: A Brief Overview of Their Diverse Applications", Jang and Kim, J Microbiol (2018) 56(4) pp 209-216); nucleic acid testing (such as nucleic

acid sequencing, and / or PCR, see for example "Routine Ribosomal PCR and DNA Sequencing for Detection and Identification of Bacteria", Kemp et al., Future Microbiol (2010) 5(7) pp 1101-1107).

[0070] In certain embodiments, the determination of the presence of an unwanted organism in the microbial composition may comprise the identification of cellular products in the cultured microbial composition, preferably cellular products not produced by the therapeutic bacteria and / or which are indicative of the presence of specific pathogenic bacteria such as pathogenic organisms from the *Clostridia* and *Salmonella* genera and the *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* species. For example, the analysis may comprise the identification of metabolites such as short chain fatty acids. Additionally or alternatively, the analysis of the microbial composition may comprise the identification of polypeptides.

[0071] The identification of such compounds may be achieved through the use of any technique or apparatus familiar to those skilled in the art, for example GC-MS or MALDI-TOF-MS analysis.

[0072] In embodiments, the process is sensitive enough to differentiate between an unacceptable level of unwanted organisms and a *de minimis* level of unwanted organisms, wherein said unacceptable level is predetermined, for example said unacceptable level may be predetermined by the FDA. In this embodiment, unnecessary waste of useful microbial compositions is avoided, for example because the user is able to determine whether the amount of unwanted organism(s) in the microbial composition is unsafe according to predetermined safety regulations.

Kits

According to a further aspect of the invention, there is provided a kit comprising an agent which selectively inhibits a first bacterial population and instructions to use the agent in the process as hereinbefore described.

Method of preparing a pharmaceutical composition

[0073] The invention also provides a method of preparing a pharmaceutical composition comprising therapeutic bacteria, comprising a step of testing for unwanted organisms by a process according to the invention. Such a method may comprise the steps of (a) culturing therapeutic bacteria, (b) performing a process according to the invention on at least a fraction of the culture of step (a) and (c) formulating the cultured therapeutic bacteria of step (a) into a pharmaceutical composition. In some embodiments, a process according to the invention may be performed following formulation of the pharmaceutical composition.

[0074] In some embodiments, a pharmaceutical composition is prepared, and tested in accordance with the invention, wherein a sample of a bulk therapeutic bacteria culture is tested and, if an acceptable level (preferably, a predefined acceptable level) of unwanted organisms is found, the bulk therapeutic bacteria culture is used in the formulation of a pharmaceutical composition (e.g. by admixing with a pharmaceutically-acceptable excipient). If greater than an acceptable level of

unwanted organisms is found (preferably, a predefined acceptable level), the bulk therapeutic bacteria culture is not used in the formulation of a pharmaceutical composition, for example the bulk therapeutic bacteria culture may be destroyed. Preferably, the (e.g. predefined) acceptable level is (i) less than 10^3 CFU/g or CFU/ml total aerobic microbial content (excluding the therapeutic bacteria), or (ii) less than 10^2 CFU/g or CFU/ml total combined yeasts and mould count; preferably both (i) and (ii). More preferably, the predefined acceptable level also requires the absence of any *Escherichia coli* bacteria (e.g. the culture does not comprise detectable *Escherichia coli* bacteria).

[0075] In an embodiment, the sample of a bulk therapeutic bacteria culture may comprise less than 1% by volume of the total bulk therapeutic bacteria culture, for example less than 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% by volume. In an embodiment, the sample of a bulk therapeutic bacteria culture may comprise less than 10% by volume of the total bulk therapeutic bacteria culture, for example less than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% by volume. In a preferred embodiment, the sample of a bulk therapeutic bacteria culture that is tested is not used in the formulation of a pharmaceutical composition. In an embodiment, the bulk therapeutic bacteria culture is at least 1 litre, 2 litres, 3 litres, 4 litres, 5 litres, 6 litres, 7 litres, 8 litres, 9 litres, or 10 litres. In an embodiment, the bulk therapeutic bacteria culture is at least 100 litres, 200 litres, 300 litres, 400 litres, 500 litres, 600 litres, 700 litres, 800 litres, or 900 litres. In an embodiment, the bulk therapeutic bacteria culture is at least 1,000 litres, 2,000 litres, 3,000 litres, 4,000 litres, 5,000 litres, 6,000 litres, 7,000 litres, 8,000 litres, 9,000 litres, or 10,000 litres.

The phage composition

[0076] The invention also provides compositions comprising one or more phages, optionally in combination with a stabiliser, preservative and / or additive. In one embodiment, the composition comprises a plurality of phages, for example 2, 3, 4, 5, 6, 7, 8, 9, 10 or greater than 10 phages may be utilised.

[0077] The one or more phages may be any phage described herein. The one or more phages may be naturally occurring or non-naturally occurring phages, for example the one or more phages may be genetically engineered or genetically modified. In an embodiment, the composition may comprise a mixture of naturally occurring phages and non-naturally occurring phages. Alternatively / in addition, the composition may comprise one or more phages that have been genetically engineered to be more or less specific for one or more therapeutic bacteria. The composition may comprise at least one phage that is specific for a Gram positive bacterium, and optionally does not comprise phages specific for a Gram negative bacterium. The composition may comprise at least one phage that is specific for a Gram negative bacterium, and optionally does not comprise phages specific for a Gram positive bacterium. The composition may comprise at least one phage having a DNA genome.

[0078] The composition may comprise one or more phages specific for one therapeutic bacterium, and optionally does not comprise any phages specific for any other therapeutic bacterium. The composition may comprise one or more phages, wherein each phage is specific for one therapeutic bacterium, and optionally each phage is specific for a different therapeutic bacterium. The composition may comprise one or more phages specific for one genus of therapeutic bacteria or one species of therapeutic bacteria. The composition may comprise one or more phages specific for one strain of therapeutic bacteria. In embodiments, the composition does not comprise more than one phage specific for the same genus of therapeutic bacteria. In embodiments, the composition does not comprise more than one phage specific for the same species of therapeutic bacteria. In embodiments, the composition does not comprise more than one phage specific for the same strain of therapeutic bacteria. The composition may comprise phages specific for more than one strain of therapeutic bacteria, and optionally may comprise phages specific for more than one strain of therapeutic bacteria of the same species. The composition may comprise phages specific for more than one species of therapeutic bacteria of the same genus.

[0079] The composition may comprise one or more phages specific for enterococcal bacteria, and optionally said composition does not comprise any phages that are not specific for enterococcal bacteria. Alternatively, the composition may comprise one or more phages specific for enterococcal bacteria and one or more phages specific for one or more non-enterococcal therapeutic bacteria.

[0080] As disclosed herein, when the therapeutic bacteria are enterococcal bacteria, particularly suitable phages include NCIMB 43666 phage and NCIMB 43667 phage, both of which are specific for enterococcal bacteria, particularly *Enterococcus gallinarum* strain NCIMB 42488. In an embodiment, the composition comprises NCIMB 43666 phage, NCIMB 43667 phage, or both. It has been found that these phages are highly specific for *Enterococcus gallinarum* strain NCIMB 42488 and do not affect other, unwanted microorganisms that may be present in the medium. These phages are therefore suitable agents for specifically inhibiting the growth of *Enterococcus gallinarum* strain NCIMB 42488.

[0081] In an embodiment, the composition comprises a phage that has a DNA genome which is at least 85% identical to SEQ ID NO: 1 and/or SEQ ID NO: 2. The composition may comprise a phage which is functionally equivalent to a phage having the genome sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2.

[0082] In an embodiment, the composition comprises a phage that has a DNA genome which is at least 85% identical to SEQ ID NOs: 3 to 8 or 3 to 68 and/or SEQ ID NOs: 69 to 74 or 69 to 2626. The composition may comprise a phage which is functionally equivalent to a phage having the genome sequence set out in SEQ ID NOs: 3 to 8 or 3 to 68 or SEQ ID NOs: 69 to 74 or 69 to 2626.

EXAMPLES

[0083] The present invention is now described in detail by reference to the following examples, which are provided by way of illustration only and should not be considered as in any way restricting the spirit and scope of the present invention, which is to be determined solely by reference to the appended claims.

[0084] The Examples make reference to the attached drawings, in which:

[0085] Figure 1 shows gel electrophoresis results for restriction fragment length polymorphism analysis of phage and bacterial DNA;

[0086] Figure 2 shows the putative gene analysis of NCIMB 43667.

[0087] The drawings are present for the purposes of illustration only and the present invention is not to be construed as being limited by the drawings.

Example 1

[0088] In the experiments described in Example 2 below, one of the phages used is the NCIMB 43667 phage. This phage was analysed in a number of ways.

[0089] 7 mL of sterile NCIMB 43667 phage lysate was used to extract the phage DNA. A few drops of chloroform were added to the lysate and the mix was centrifuged at 3000 x g for 5 min. The supernatant was filtered through a 0.2 µm syringe filter. 5 mL of the filtrate was collected and mixed with 43 µL of 1 mg/mL DnaseI and 39 µL of 5 mg/mL Rnase A. The mixture was incubated for 30 min at 37°C. Then 91.2 µL of 20 % SDS and 23.8 µL proteinase K at 15 mg/mL were added. The mixture was further incubated for 30 min at 37°C. The entire volume was transferred into a new 15 mL centrifuge tube. The solution was mixed 1:1 with phenol / chloroform (pH 8). The phases were separated by centrifugation for 5 min at 1500 x g. The aqueous phase was removed into a new 15 mL tube and the step was repeated. The aqueous phase was removed and extracted once with an equal volume of chloroform. The upper phase was removed after centrifugation for 5 min at 6000 x g. 10 µL of 3M sodium acetate and 1 volume of 100 % isopropanol were added. The DNA was left to precipitate at room temperature for 20 min and was then centrifuged at 14000 x g for 20 min. The DNA pellet was washed with 70 % ethanol. The ethanol was removed and the DNA pellet was dried at room temperature for 10 min. The DNA was resuspended in 500 µL 5 mM Tris-HCl, pH 8.5.

[0090] To determine the concentration, the DNA was diluted 1:10 in water and the absorbance at 260 nm and 280 nm was determined. The results of the determination are set out in Table 1 below.

Table 1. NCIMB 43667 phage DNA concentration determination

Description	A ₂₆₀ (1:10 dilution)	A ₂₈₀ (1:10 dilution)	A ₂₆₀ /A ₂₈₀	Concentration	Yield
NCIMB 43667 phage DNA	0.158	0.084	1.88	79 ng/μL	39.5 μg

[0091] To make sure that phage, and not bacterial, DNA was isolated, Restriction Fragment Length Polymorphism (RFLP) analysis was performed. To provide a control, total bacterial DNA from *E. gallinarum* was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol. A bacterial pellet from a 5 mL overnight culture was used for extraction. The concentration of bacterial DNA was determined as described above. The results are shown in Table 2 below.

Table 2. *Enterococcus gallinarum* DNA concentration determination

Description	A ₂₆₀ (1:5 dilution)	A ₂₈₀ (1:5 dilution)	A ₂₆₀ /A ₂₈₀	Concentration	Yield
<i>E. gallinarum</i> DNA	0.124	0.051	2.43	30.75 ng/μL	6.2 μg

[0092] The phage DNA was digested with various restriction enzymes. 500 ng of DNA was used in each reaction. 1 unit of each enzyme was used in the reaction in a volume of 20 μL, as shown in Table 3 below.

Table 3. Restriction Enzyme Digests of Phage and Bacterial DNA

DNA	Enzyme	Buffer	Reaction Description	Incubation
NCIMB 43667	EcoRI	Unique	20 μL reaction volume, phage or host DNA (500 ng), 2 μL 10 x buffer, 10 units of enzyme, water to 20 μL	37°C, 1 h
	EcoRV	Buffer R		
	HindIII	Buffer R		
	HpaII	Tango		
	Sall	Buffer O		
	NotI	Buffer O		
	BglII	Buffer O		
	BamHI	Unique		
<i>E. gallinarum</i>	HindIII	Buffer R		

[0093] After incubation, a DNA loading dye was added and the samples were subjected to gel electrophoresis on 1% agarose gel stained with 1 μg/mL ethidium bromide. The enzymes EcoRI and EcoRV gave distinct DNA fragment patterns for phage DNA. Enzyme HindIII was used to cut *E.*

gallinarum DNA. The restriction enzyme digest pattern for bacterial DNA was clearly different from that obtained for phage DNA. This is shown in Figure 1. The RFPL analysis prove that the phage DNA was dsDNA. The phage DNA preparation was of good quality and was free of bacterial DNA contamination. The DNA was suitable for sequencing.

[0094] The DNA was then sequenced using conventional protocols for producing DNA libraries. Illumina next-generation sequence data at a minimum coverage of 30x were produced. 224 contigs were obtained of which only three were longer than 1,000 bp. The largest contig representing the DNA sequence of the phage was 57201 bp long, with the mean coverage of 1,756.31. The complete genome sequence is given in SEQ ID NO: 1 in the sequence listing, as described above.

[0095] The DNA sequence had: an A content of 31.51 %; a C content of 19.56 %; a G content of 21.95%; a T content of 26.98 %; and a CG percentage of 41.51%.

[0096] Putative genes were annotated using the PHASTER web server for the identification and annotation of prophage sequences within bacterial genomes and plasmids. The termini of the DNA sequence were not analysed. It was therefore not possible to determine whether the phage genome was linear or circular. The putative gene analysis is shown in Figure 2.

[0097] The 57,202 bp sequence of NCIMB 43667 phage was searched for sequences similar to those in the NCBI databases using the BlastN search tool with a highly similar sequences search algorithm (megablast). The sequences which showed the highest similarity to the NCIMB 43667 sequence were the sequences of Enterococcus phage vB_EfaS_Ef7.1 and Enterococcus phage VD13. The megablast hit table for highly similar sequences is shown in Table 4 below.

Table 4 Megablast Hit Table with Highly Similar Sequences to NCIMB 43667 Phage Sequence

Description (all complete genome) (E = Enterococcus)	Max scores	Total Score	Query cover	E value	Ident.	Accession
E phage vB_EfaS_Ef7.1	361	626	2%	2.00E-93	74.63%	MK721194.1
E phage VD13	294	294	0%	2.00E-73	81.67%	NC_041861.1
E phage VD13	294	294	0%	2.00E-73	81.67%	KJ094032.2
E phage VD13	294	294	0%	2.00E-73	81.67%	KJ127303.1
E phage SAP6	278	278	1%	2.00E-68	73.24%	NC_041960.1
E phage SAP6	278	278	1%	2.00E-68	73.24%	JF731128.1
E phage IME-EF1	265	428	1%	2.00E-64	73.15%	NC_041959.1
E phage IME-EF1	265	428	1%	2.00E-64	73.15%	KF192053.1
E phage vB_EfaS_IME198	174	174	0%	3.00E-37	78.45%	KT932699.1
E phage vB_EfaS_Ef2.2	163	231	0%	7.00E-34	77.86%	MK721189.1

[0098] The characteristics of NCIMB 43667 phage shows that it is likely to be useful in the process of the present invention. This is shown by the following Example 2.

Example 2

[0099] As demonstrated in WO 2017/085520, strains of the species *Enterococcus gallinarum* are effective for treating and preventing cancer. Thus, these species are therapeutic bacteria as defined above. A particular strain of *Enterococcus gallinarum* has been deposited under the accession number NCIMB 42488 and is described in more detail in WO 2017/085520.

[0100] A microbial composition containing NCIMB 42488 was supplied in capsules. Each capsule contained about 5×10^{10} CFU of *E. gallinarum*. The capsules were stored at $5 \pm 3^\circ\text{C}$ before use.

[0101] The experiments set out below comprise the use of two bacteriophages, NCIMB 43666 and NCIMB 43667.

Experimental details and results

Double agar overlay assay for determination of phage titre

[0102] The phage detection process is based on the ability of phages to create clear zones (plaques) on a lawn of host cells. A double agar overlay assay was applied in this study.

[0103] For phage plating, 100 μL of phage dilution in SM buffer is mixed with 500 μL of an overnight culture of NCIMB 42488 and incubated for 15 min at 37°C . The phage-host mixture is added to 3 mL of top agar which had been warmed to 42°C and the evenly distributed over CASO agar. The plates are incubated at 37°C for 16-48 h and inspected for plaque formation. The phage titre was determined from plates with plaque numbers between 10 and 300. The number of plaque forming units in 1 mL was calculated using the equation:

$$\bar{c} = \frac{\sum c}{n_1 * 1 + n_2 * 0,1}$$

where:

- $\bar{c}$ weighted average of the plaques number
- $\sum c$ sum of the plaques from all plates used for calculations
- n_1 number of plates with the lowest dilution
- n_2 number of plates with the next highest dilution.

[0104] The number of plaque forming units in 1 ml was calculated using the equation:

$$\text{pfu / mL} = \text{weighted average } c * 10 * \text{reciprocal of the lowest dilution}$$

Production of NCIMB 43667 phage lysate and quality control

[0105] The bulk volume of NCIMB 43667 phage lysate was produced to be further used for production of liquid and solid media containing phage cocktail against NCIMB 42488. An exponentially growing culture of NCIMB 42488 was prepared by inoculation of 95 mL CASO bouillon with 5 mL overnight culture of the strain. The diluted culture was further incubated for 1 h at 37°C and infected with 10 µL NCIMB 43667 phage. The NCIMB 43667 phage inoculum used for infection was prepared from a filter-sterilized sample. After infection, the culture was further incubated without agitation for 16 h until lysed. The lysate was pooled, 1 mL chloroform was added, mixed using vortex and centrifuged at 3000 x g for 5 min. The supernatant was filter-sterilized. The titre of the phage was determined as described above. The results are given in Table 5.

Table 5 – NCIMB 43667 phage titre determination on NCIMB 42488

NCIMB 43667 phage titre determination on NCIMB 42488			
Dilution	No. of plaques		
	Replicate 1	Replicate 2	Mean
10 ⁻⁶	228	232	230
10 ⁻⁷	27	28	27.5
10 ⁻⁸	5	3	4
Titre (pfu/mL)	2.3 x 10 ⁹		
Lowest acceptable titre (pfu/mL)	≥1.8 x 10 ⁹		
Evaluation	Complies		

[0106] The sterility of the phage was tested using the spread plate method. 100 µL of the NCIMB 43667 solution was spread-plated on CASO agar in duplicate and incubated for 48 h at 32.5 ± 2.5°C. The results are given in Table 6. The NCIMB 43667 solution was stored in two 40 mL aliquots at 5±3°C until use.

Table 6 – NCIMB 43667 Sterility Testing

Phage Lysate	Sample	Incubation	Acceptance Criteria	Results	Evaluation
NCIMB 43667	100 µL of phage solution replicate 1	48 h at 32.5± 2.5°C on CASO agar	No growth of microorganisms	No growth	Complies
	100 µL of phage solution replicate 2			No growth	Complies

[0107] The criterion of the lowest acceptable concentration of the phage preparation was set as not less than 50% of the titre determined previously in the method development study.

Production of NCIMB 43666 phage lysate and quality control

[0108] The bulk volume of NCIMB 43666 phage lysate was produced to be further used for production of liquid and solid media containing phage cocktail against NCIMB 42488. The decimal dilution series of NCIMB 43666 phage was prepared in SM buffer. The dilutions 10^{-3} , 10^{-4} and 10^{-5} were plated in a volume of 100 µL together with 0.5 mL overnight culture of NCIMB 42488. LB agar plates supplemented with 5mM $MgSO_4$ + 5mM $CaCl_2$ were used. Each dilution was plated in quadruplicate using the method described above. The plates were incubated for 16-48 h at 37°C. Plates showing confluent lysis were washed with 5 mL SM buffer / plate for 4 h on a 3D rotator. The lysate was pooled, 0.5 mL chloroform was added, mixed using vortex and centrifuged at 3000 x g for 5 min. The supernatant was filter sterilized. The titre of the NCIMB 42488 was determined as described above (results shown in Table 7). The sterility of the phage was tested using spread plate method. A volume of 100 µL of the phage was spread-plated on CASO agar in duplicate and incubated for 48 h at 32.5 ± 2.5°C (results shown in Table 8). The phage was stored in one 20 mL aliquot at 5±3 °C until use.

[0109] The criterion of the lowest acceptable concentration of the phage preparation was set as not less than 50% of the titre determined as set forth above.

Table 7. NCIMB 43666 phage titre determination on NCIMB 42488

NCIMB 43666 phage titre determination on NCIMB 42488			
Dilution	No. of plaques		
	Replicate 1	Replicate 2	Mean
10^{-5}	TNTC	TNTC	n.a.
10^{-6}	58	55	56.5
10^{-7}	6	3	4.5

10 ⁻⁸	0	0	0
Titre (pfu/mL)	5.7 x 10 ⁸		
Lowest acceptable titre (pfu/mL)	≥5.2 x 10 ⁸		
Evaluation	Complies		
TNTC = too numerous to count			
n.a. = not applicable			

Table 8. NCIMB 43666 sterility testing

Phage Lysate	Sample	Incubation	Acceptance Criteria	Results	Evaluation
NCIMB 43666	100 μ L of phage solution replicate 1	48 h at $32.5 \pm 2.5^\circ\text{C}$ on CASO agar	No growth of microorganisms	No growth	Complies
	100 μ L of phage solution replicate 2			No growth	Complies

Production of NCIMB 43667 and NCIMB 43666 phage cocktail

[0110] The two phage stock solutions were mixed in equal volumes directly before use. The final concentration of the phages in the cocktail was 2.85×10^8 and 1.2×10^9 PFU/ml of NCIMB 43666 and NCIMB 43667 respectively. The phage cocktail was used to cover the agar plates used for the detection of contaminant microorganisms.

Preparation of test strains

[0111] Test strains were prepared as cryo stocks with 43% glycerol and are stored at $\leq -64^\circ\text{C}$. The cell counts were determined on an approved batch of media. Dilutions in peptone water were prepared to obtain <100 CFUs in 100 μ L as indicated in Table 9 below.

Table 9. Growth promotion of solid media – preparation of test strains

Strain	Given cell number (cfu/mL)	Dilution in peptone water buffer	Calculated number of cfu / 100 μ L
<i>S. aureus</i> ATCC 6538	2.8×10^5	1 : 300	93
<i>B. cereus</i> ATCC 11778	3.8×10^5	1 : 400	95
<i>C. sporogenes</i> ATCC 19404	1.1×10^5	1 : 120	92

Growth promoting properties of solid media with phages

[0112] The control contaminant microorganisms were diluted to less than 10^3 CFU /mL in

buffered peptone water and 100 µL of the dilutions were spread-plated on CASO or Columbia blood agar. The plates were covered with 100 µL phage cocktail and allowed to dry before plating the control microorganisms. Each strain preparation was plated in duplicate. The Columbia agar plates with *C. sporogenes* ATCC 19404 were incubated at $32.5 \pm 2.5^\circ\text{C}$ for ≤ 48 h in anaerobic atmosphere created with the Anaerobic Jar GasPack system. The CASO agar plates with *S. aureus* ATCC 6538 and *B. cereus* ATCC 11778 were incubated for ≤ 18 h at $32.5 \pm 2.5^\circ\text{C}$ aerobically. The acceptance criteria and results are presented in Table 10.

Table 10. Growth promotion of solid media

Description of the growth promotion test	Given number of CFU	Number of CFU repl. 1 / repl. 2		Mean number of CFU recovered	Acceptance criteria	Results	Evaluation
< 100 CFU of the <i>S. Aureus</i> on CASO agar covered with phage cocktail	93	51	58	54.5	The mean number of CFU must not differ greater than a factor of 2 from the calculated value; reduction factor [R]: $0.5 \leq [R] \leq 2$	Reduction factor: 1.7	Complies
< 100 CFU of <i>B. Cereus</i> on CASO agar covered with phage cocktail	95	80	82	81		Reduction factor 1.17	Complies
< 100 CFU of <i>C. sporogenes</i> on Columbia agar covered with phage cocktail	92	123	108	115.5		Reduction factor: 0.8	Complies

Growth promoting properties of liquid media, suitability of the test method and test for the absence of contaminating microorganisms

Preparation of the sample

[0113] Each tested capsule of NCIMB 42488 drug product was removed from the blister aseptically using flamed tweezers in laminar air flow cabinet and suspended in 10 mL buffered peptone water. The capsule shell was dissolved after about 30 min incubation and the content was homogenized by vortexing. The suspension was stored not longer than 8 h at $2-8^\circ\text{C}$ before use. For testing, the entire

volume of the suspension (10 mL) was withdrawn. The residual volume was next collected by single wash step with 10 mL of appropriate cultivation media.

Test procedure

[0114] The test for the detection of contaminant bacteria was performed in two steps. The first step was the enrichment of the potential contaminant strain in liquid culture together with the sample and bacteriophage cocktail active against *E. gallinarum* NCIMB 42488. The second step was the subculture of the enrichments on agar plates previously covered with phage cocktail.

[0115] Depending on the tested strain, the enrichments were made in CASO broth (*S. aureus* ATCC 6538 and *B. cereus* ATCC 11778) or in RCM broth (*C. sporogenes* ATCC 19404). Both media were supplemented with 5 mM MgSO₄. The CASO cultures were incubated for 24 h at 32.5 ± 2.5°C without shaking and the RCM cultures were incubated for 48 h under the same conditions. Each enrichment consisted of:

- 990 mL broth (RCM or CASO);
- 1 mL NCIMB 43667 phage;
- 0.2 mL NCIMB 43666 phage;
- 10 mL sample (1 capsule in peptone water) or 10 mL peptone water; and
- 100 µL of the test strain where appropriate (less than 100 CFU).

[0116] After enrichment, subcultures were made on CASO agar (*S. aureus* ATCC 6538 and *B. cereus* ATCC 11778 and Columbia blood agar (*C. sporogenes* ATCC 19404). The agar plates were covered with 100 µL phage cocktail each by spread-plating before subculturing and allowed to dry in a laminar flow cabinet. The subcultures were made by 10 µL dilution streak in triplicates from each enrichment. The CASO agar plates were incubated at 32.5 ± 2.5°C for 24 h aerobically and Columbia blood agar plates for 48 h in anaerobic atmosphere. The testing was conducted according to the following scheme (Table 11):

Table 11. Testing scheme for the determination of growth promoting properties of liquid media, suitability of the test method and test for the absence of contaminating microorganisms

Pos	Enrichment	Funtion
1	1 capsule NCIMB 42488 + phages in CASO broth	Testing for the absence of <i>S. aureus</i> ATCC 6538
2	1 capsule NCIMB 42488 + phages in CASO broth	
3	1 capsule NCIMB 42488 + phages in CASO broth	
4	1 capsule NCIMB 42488 + phages in CASO broth	Testing for absence of <i>B. cereus</i> ATCC 11778
5	1 capsule NCIMB 42488 + phages in CASO broth	
6	1 capsule NCIMB 42488 + phages in CASO broth	
7	1 capsule NCIMB 42488 + phages + <i>S. aureus</i> in CASO broth	Method suitability for detection of <i>S. aureus</i> ATCC 6538
8	1 capsule NCIMB 42488 + phages + <i>S. aureus</i> in CASO broth	
9	1 capsule NCIMB 42488 + phages + <i>S. aureus</i> in CASO broth	

10	1 capsule NCIMB 42488 + phage + <i>B. cereus</i> in CASO broth	Method suitability for detection of <i>B. cereus</i> ATCC 11778
11	1 capsule NCIMB 42488 + phages + <i>B. cereus</i> in CASO broth	
12	1 capsule NCIMB 42488 + phages + <i>B. cereus</i> in CASO broth	
13	10 mL peptone water phages in CASO broth	Negative control in the test for detection of <i>S. aureus</i> ATCC 6538 and <i>B. cereus</i> ATCC 11778
14	10 mL peptone water + phages + <i>S. aureus</i> in CASO broth	Growth promoting properties of the media
15	10 mL peptone water + phages + <i>B. cereus</i> in CASO broth	
16	1 capsule NCIMB 42488 + phages in RCM, anaerobic	Testing for the absence of <i>C. sporogenes</i> ATCC 19404
17	1 capsule NCIMB 42488 + phages in RCM, anaerobic	
18	1 capsule NCIMB 42488 + phages in RCM	
19	1 capsule NCIMB 42488 + phages + <i>C. sporogenes</i> in RCM	Method suitability for detection of <i>C. sporogenes</i> ATCC 19404
20	1 capsule NCIMB 42488 + phages + <i>C. sporogenes</i> in RCM	
21	1 capsule NCIMB 42488 + phages + <i>C. sporogenes</i> in RCM	
22	10 mL peptone water + phages in RCM	Negative control in the test for detection of <i>C. sporogenes</i> ATCC 19404
23	10 mL peptone water + phages + <i>C. sporogenes</i> in RCM	Growth promoting properties of the media

Growth-promoting properties of the liquid media and negative controls

[0117] The growth promoting properties of the liquid media were tested as described above. The diluent (peptone water) was used in place of the sample in the enrichment of the contaminant strains. The results are presented in Table 12. Both batches of CASO broth and RCM broth used in this study showed growth promoting properties for the specified microorganisms and were approved for use in this study. The negative controls proved that the CASO broth batch was free of *S. aureus* ATCC 6538 and *B. cereus* ATCC 11778 and RCM broth was free of *C. sporogenes* ATCC 19404.

Table 12. Growth promoting properties of liquid media and negative controls

Test for specified microorganisms – Growth promoting properties of liquid media and negative controls							
Enrichment				Subculture and detection of test microorganisms			
Amount of NCIMB 42488	Spiked micro-organism	Phage Cocktail	Incuba'n	Incuba'n	Detection of <i>S. aureus</i> ATCC 6538	Detection of <i>B. cereus</i> ATCC 11778	Detection of <i>C. sporogenes</i> ATCC 19404
0	< 100 CFU of <i>S. aureus</i> ATCC	0.2 mL of NCIMB 43666 and 1 mL	32.5°C, 24 h, CASO broth, aerobic	32.5°C, 24 h, CASO agar	Yes	n.a.	n.a.
					Yes	n.a.	n.a.
					Yes	n.a.	n.a.

	6538	of NCIMB 43667					
0	< 100 CFU <i>B. cereus</i> ATCC 11778				n.a.	Yes	n.a.
					n.a.	Yes	n.a.
					n.a.	Yes	n.a.
0	None – negative control				n.a.	n.a.	Yes
					n.a.	n.a.	Yes
		n.a.	n.a.	Yes			
0	< 100 CFU <i>C. sporogenes</i> ATCC 19404	0.2 mL of NCIMB 43666 and 1 mL of NCIMB 43667	32.5°C, 48 h in RCM, anaerobic	32.5°C, 48 h, Columbia blood agar with phages	n.a.	n.a.	Yes
					n.a.	n.a.	Yes
					n.a.	n.a.	Yes
0	None – negative control				n.a.	n.a.	No
					n.a.	n.a.	No
					n.a.	n.a.	No

Method suitability for detection of *S. aureus*, *B. cereus* and *C. sporogenes* in NCIMB 42488

[0118] The test strains were prepared as described above. The recovery of a low number of the test strains in the presence of the sample was tested to prove the method suitable for detection. The test was performed as described above. For each tested strain, the enrichment step was repeated three times. The subculture step after each enrichment was performed in triplicate also. The results are presented in Table 13.

Table 13. Suitability of the test method for detection of the test strains

Test for specified microorganisms – suitability of the test method						
Enrichment			Subculture and detection of test microorganisms			
Amount of sample NCIMB 42488 spiked with test strain	Phage cocktail	Incubation	Incubation	Recovery and detection of <i>S. aureus</i> ATCC 6538 (93 CFU)	Recovery and detection of <i>B. cereus</i> ATCC 11778 (95 CFU)	Recovery and detection of <i>C. sporogenes</i> ATCC 19404 (92 CFU)
1 capsule (replicate 1)	0.2 mL of NCIMB 43666 and 1 mL of NCIMB 43667	32.5°C, 24 h in CASO broth or 48 h in RCM broth	32.5°C, 24 h on CASO agar or 32.5°C, 48 h on Colombia blood agar	Yes	Yes	Yes
				Yes	Yes	Yes
				Yes	Yes	Yes
1 capsule (replicate 2)				Yes	Yes	Yes
				Yes	Yes	Yes
				Yes	Yes	Yes
1 capsule (replicate 3)				Yes	Yes	Yes
				Yes	Yes	Yes
				Yes	Yes	Yes

[0119] The method was proved to be suitable for the detection of *S. aureus* ATCC 6538, *B. cereus* ATCC 11778 and *C. sporogenes* ATCC 19404 in the presence of 1 capsule of the NCIMB 42488 containing around 5×10^{10} CFU/capsules. Colonies visually identified as *S. aureus* were picked in order to confirm their identity via the Staphaurex™ latex agglutination test, according to the manufacturer's instructions. *S. aureus* shows a positive reaction in the latex test while NCIMB 42488 shows a negative reaction. The detection of *B. cereus* was confirmed by colony morphology and microscopically after Gram staining. The presence of Gram-positive rods, endospores forming confirmed the detection of *B. cereus*.

[0120] The occurrence of characteristic colonies on Columbia blood agar and detection of rod-shaped Gram-positive cells (with or without endospores), giving negative result in catalase test confirmed the detection of *C. sporogenes*. The characteristic colonies were large and flat with a rhizoid margin and a raised centre. The margins of the colony were dry and rough.

Test for the presence of *S. aureus*, *B. cereus* and *C. sporogenes* in NCIMB 42488.

[0121] To determine the presence of *S. aureus*, *B. cereus* and *C. sporogenes* in the NCIMB 42488 drug product, the test was performed as described above. Three capsules were tested separately for the absence of each test strain (3 enrichments per strain). The subcultures from each enrichment were made in triplicate. The absence of test microorganisms was stated when no colonies of the test microorganism were found after subculture on any of the plates prepared. The results are presented in Table 14. None of the above-mentioned contaminant strains was found in the NCIMB 42488 drug product capsules.

Table 14. Detection of specified microorganisms in NCIMB 42488 drug product sample

Test for specified microorganisms – detection of contaminating microorganisms						
Enrichment			Subculture and detection of test microorganisms			
Amount of sample	Phage cocktail	Incubation	Incubation	Detection of <i>S. aureus</i>	Detection of <i>B. cereus</i>	Detection of <i>C. sporogenes</i>
1 capsule Replicate 1	0.2 mL of NCIMB 43666 and 1 mL of NCIMB 43667	32.5°C, 24 h in CASO broth or 48 h in RCM broth	32.5°C, 24 h on CASO agar or 32.5°C, 48 h on Columbia blood agar	No	No	No
				No	No	No
				No	No	No
1 capsule Replicate 2				No	No	No
				No	No	No
				No	No	No
1 capsule Replicate 3				No	No	No
				No	No	No
				No	No	No

Conclusion

[0122] The described method is suitable for the detection of *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778 and *Clostridium sporogenes* ATCC 19404 in 1 capsule of *E. gallinarum*

NCIMB 42488 drug product (CFU/capsule) according to the demands of Ph. Eur. 2.6.38. The use of the phage cocktail allows for sufficient reduction of *E. gallinarum* NCIMB 42488 cells to allow for detection of contaminant strains.

[0123] None of the abovementioned contaminant strains were found in the *E. gallinarum* NCIMB 42488 drug product capsules.

Example 3

Experimental details and results

Double agar overlay assay for determination of phage titre

[0124] The phage detection process is based on the ability of phages to create clear zones (plaques) on a lawn of host cells. A double agar overlay assay was applied in this study.

[0125] For phage plating, 100 µL of phage dilution in SM buffer is mixed with 500 µL of an overnight culture of NCIMB 42488 and incubated for 15 min at 37°C. The phage-host mixture is added to 3 mL of top agar which had been warmed to 42°C and the evenly distributed over CASO agar. The plates are incubated at 37°C for 16-48 h and inspected for plaque formation. The phage titre was determined from plates with plaque numbers between 10 and 300. The number of plaque forming units in 1 mL was calculated using the equation:

$$\bar{c} = \frac{\sum c}{n_1 * 1 + n_2 * 0,1}$$

where:

$\bar{c}$ weighted average of the plaques number

$\sum c$ sum of the plaques from all plates used for calculations

n_1 number of plates with the lowest dilution

n_2 number of plates with the next highest dilution.

[0126] The number of plaque forming units in 1 ml was calculated using the equation:

$$\text{pfu / mL} = \text{weighted average } c * 10 * \text{reciprocal of the lowest dilution}$$

Production of NCIMB 43667 phage lysate and quality control

[0127] The 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ dilutions of NCIMB 43667 phage in SM buffer were prepared. 100 µl of each dilution was plated in 3ml molten top agar in triplicates with 500 µl overnight culture of NCIMB 42488 (in CASO broth, 37 °C no agitation). Supplemented LB agar was used. The plates were incubated 18h at 37 °C. The plates showing confluent lysis were washed with 5 ml SM buffer/ plate for 4 h on 3D rotator. The phage lysate was pooled, and 0.5 ml chloroform was added. The mix was

centrifuged at 3000 x g for 5 min. The supernatant was filter sterilized and sterile DMSO was added to a final concentration of 7 %. Aliquots of 0.5 mL were prepared and were stored at $\leq -64^{\circ}\text{C}$ until use.

[0128] To determine the phage titre in the NCIMB 43667 phage stock, one WCB was thawed. Phage titre was determined on CASO agar by plating 100 μL dilutions of the phage in SM buffer with 500 μL overnight culture of the NCIMB 42488. Results are in Table 15 below.

Table 15. Titre determination of NCIMB 43667 phage suspension

Dilution	NCIMB 43667 on NCIMB 42488		
	No. of plaques		Mean
10^{-4}	n.a	n.a.	n.a
10^{-5}	220	218	219
10^{-6}	16	23	19.5
10^{-7}	2	2	2
10^{-8}	0	0	-
10^{-9}	0	0	-
Titre [pfu/ml]	2.2×10^8		

Production of NCIMB 43666 phage stock solution

[0129] The NCIMB 43666 phage stock solution was prepared using the procedure described above. Aliquots of 0.5 mL were prepared and were stored at $\leq -64^{\circ}\text{C}$. Phage titre was determined on CASO agar by plating 100 μL dilutions of the phage in SM buffer with 500 μL overnight culture of the NCIMB 42488. Results are shown below in Table 16.

Table 16. Titre determination of NCIMB 43666 phage suspension

Dilution	NCIMB 43666 on NCIMB 42488		
	No. of plaques		Mean
10^{-4}	n.a	n.a.	n.a
10^{-5}	454	>500	n.a
10^{-6}	44	61	52.5
10^{-7}	5	5	5
10^{-8}	0	0	-
10^{-9}	0	0	-
Titre [pfu/ml]	5.2×10^8		

Production of NCIMB 43667 and NCIMB 43666 phage cocktail

[0130] Both phage stock solutions were mixed 1:1 directly before use. The final concentration of the phages was 2.6×10^6 PFU of NCIMB 43666 and 1.1×10^6 PFU of NCIMB 43667 in 10 μ l cocktail.

Determination of phage activity in liquid cultures containing NCIMB 42488

[0131] An overnight culture of NCIMB 42488 was prepared in CASO broth. The culture was diluted 1:100 and 100 μ l aliquots in sterile 96-well titre plate were prepared. Each aliquot was spiked with 10 μ l containing 1, 10, 100, and 1000 NCIMB 43667 and NCIMB 43666 each and in combination (as 1:1 cocktail). Additionally undiluted phage stock solutions were tested: 2.2×10^6 PFU of NCIMB 43667 phage and 5.2×10^6 PFU NCIMB 43666. The phages were diluted to the desired concentration in SM buffer. The kinetics of the bacterial culture turbidity changes (OD_{620nm}) were measured during 6 h incubation in 30 min intervals.

[0132] Phage NCIMB 43667 showed significantly better performance against NCIMB 42488 than phage NCIMB 43666. Phage NCIMB 43666 was not able to lyse the liquid cultures irrespective of the phage amount spiked. Slightly slower growth of NCIMB 42488 was observed when the culture was spiked with 5.2×10^6 PFUs of NCIMB 43666. Cultures spiked with NCIMB 43667 phage were completely lysed after about 4h even when very low numbers of phage were initially in the culture. Cultures spiked with high number of NCIMB 43667 phage (2.2×10^6 PFU) did not show growth at all. These data indicate that the NCIMB 43667 phage replicates via lytic cycle on NCIMB 42488 and is a very good candidate to be used as an agent for the neutralization of *E. gallinarum* in microbial examination of compositions containing NCIMB 42488. No evidence of improvement in NCIMB 42488 killing activity was observed when phage cocktail containing both phages was used. Complete lysis and no further growth of bacteria in the presence of NCIMB 42488 specific phages indicate that the sample tested was microbiologically pure.

Table 17. Clarification of NCIMB 42488 liquid cultures infected with NCIMB 43667, NCIMB 43666 phages each and in combination – mean values from 3 replicates

Mean OD620 changes of NCIMB 42488 culture infected with NCIMB 43667 phage													
No of infecting phages	Time after infection [min]												
	0	30	60	90	120	150	180	210	240	270	300	330	360
0	0,05	0,05	0,06	0,07	0,09	0,14	0,21	0,46	0,56	0,57	0,57	0,57	0,55
1	0,05	0,05	0,06	0,08	0,10	0,14	0,22	0,25	0,23	0,21	0,20	0,20	0,19
10	0,05	0,05	0,06	0,07	0,09	0,13	0,17	0,09	0,07	0,06	0,05	0,05	0,05
100	0,05	0,05	0,06	0,07	0,09	0,11	0,08	0,05	0,05	0,04	0,04	0,04	0,04
1000	0,05	0,05	0,06	0,07	0,08	0,07	0,05	0,05	0,04	0,04	0,04	0,04	0,04

10000	0,05	0,05	0,06	0,07	0,06	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04
2200000	0,04	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04
Mean OD620 changes of NCIMB 42488 culture infected with NCIMB 43666 phage													
0	0,05	0,05	0,06	0,07	0,09	0,14	0,20	0,42	0,54	0,56	0,56	0,54	0,53
1	0,04	0,05	0,06	0,07	0,09	0,13	0,21	0,43	0,54	0,54	0,53	0,51	0,52
10	0,05	0,05	0,06	0,07	0,09	0,13	0,20	0,42	0,56	0,57	0,57	0,56	0,54
100	0,05	0,05	0,06	0,07	0,09	0,14	0,21	0,45	0,56	0,57	0,58	0,57	0,55
1000	0,05	0,05	0,06	0,08	0,09	0,15	0,22	0,46	0,56	0,57	0,57	0,57	0,55
10000	0,05	0,05	0,06	0,08	0,09	0,14	0,21	0,45	0,55	0,55	0,55	0,54	0,53
5200000	0,05	0,05	0,05	0,06	0,07	0,09	0,12	0,23	0,34	0,49	0,44	0,45	0,46
Mean OD620 changes of NCIMB 42488 culture infected with NCIMB 43667 phage and NCIMB 43666 phage cocktail													
0	0,04	0,05	0,06	0,07	0,09	0,14	0,20	0,42	0,55	0,57	0,56	0,55	0,54
1	0,05	0,05	0,06	0,07	0,09	0,13	0,20	0,43	0,54	0,55	0,54	0,53	0,52
10	0,05	0,05	0,06	0,07	0,10	0,14	0,18	0,11	0,07	0,06	0,06	0,05	0,05
100	0,05	0,05	0,06	0,07	0,10	0,13	0,11	0,07	0,06	0,05	0,05	0,05	0,05
1000	0,05	0,05	0,06	0,07	0,09	0,09	0,06	0,05	0,05	0,05	0,04	0,04	0,04
10000	0,04	0,05	0,05	0,07	0,07	0,05	0,04	0,04	0,04	0,04	0,04	0,04	0,04

Test for enumeration of microbial contaminants

[0133] The test strains were prepared as cryo stocks with 43% glycerol and are stored at ≤ -64 °C. The cell counts were determined before. Dilutions in peptone water prepared to obtain <100 CFUs in 100 μ L as indicated in the table below.

Table 20. Preparation of test strains

Strain	MM strain No.	PRID	PCH	Given cell number in the cryostock [cfu/ mL]	Dilution in buffered peptone water	Calculated number of cfu/ 100 μ L
<i>S. aureus</i>	1178	695	6335	2.6×10^6	1:2700	96
<i>P. aeruginosa</i>	242	484	6131	3.1×10^5	1:320	96
<i>B. subtilis</i>	241	486	6336	1.6×10^5	1:170	94

[0134] To verify testing conditions, a negative control was performed using the buffered peptone water as diluent in place of the test preparation. There must be no growth of micro-organisms.

[0135] The test for growth promotion of the media was performed as follows:

(A) <100 CFU of *S. aureus* (100µl) and 10 µl of phage cocktail were spread-plated on CASO agar. Analogously *P. aeruginosa* and *B. subtilis* were tested.

(B) 100 µl buffered peptone water and 10 µl phage cocktail were spread-plated on CASO agar

(C) <100 CFU of *S. aureus* (100µl) without phages was spread-plated on CASO agar.

[0136] *P. aeruginosa* and *B. subtilis* were tested in the same way and plates A, B, and C were incubated for 3 days at 32.5 °C. Acceptance criteria were as follows:

- the counts of *S. aureus*, *P. aeruginosa* and *B. subtilis* on plates A and C should not differ more than 50 %
- the growth obtained on plate A must not differ by a factor greater than 2 from the calculated value for the stable *S. aureus*, *P. aeruginosa* and *B. subtilis* inocula
- No growth observed on plate B (negative control)

[0137] All acceptance criteria for test strains recovery were met. No significant differences were observed in the recovery of the test strains where phage cocktail was used and where phage cocktail was not added. Thus, the use of the phage cocktail has no impact on the validity of the test. Phage cocktail can be used as an agent for neutralization of NCIMB 42488 in the test for enumeration of microbial contaminants microbial compositions containing NCIMB 42488. The phage cocktail is selectively active against *E. gallinarum* and none from the test strains was affected.

Table 21. Growth promotion of the media – results

Plate	Description	Calculated number of CFU	Number of CFU recovered	Acceptance criteria	Results	Evaluation
A	<100 CFU of <i>S. aureus</i> + phage cocktail	96	108	The mean number of CFU must not differ greater than a factor of 2 from the calculated value	No reduction	Complies
B	Buffered peptone water + phage cocktail (negative control)	0	0	No microbial growth	No microbial growth	Complies

Plate	Description	Calculated number of CFU	Number of CFU recovered	Acceptance criteria	Results	Evaluation
C	<100 CFU of <i>S. aureus</i> without phages	96	118	The mean number of CFU must not differ more than 50% between plate A and C	% difference in the number of <i>S. aureus</i> recovered: 9 %	Complies
A'	<100 CFU of <i>P. aeruginosa</i> + phage cocktail	96	89	The mean number of CFU must not differ greater than a factor of 2 from the calculated value	No reduction	Complies
C'	<100 CFU of <i>P. aeruginosa</i> without phages	96	80	The mean number of CFU must not differ more than 50% between plate A' and C'	% difference in the number of <i>P. aeruginosa</i> recovered: 10%	Complies
A''	<100 CFU of <i>B. subtilis</i> + phage cocktail	94	101	The mean number of CFU must not differ greater than a factor of 2 from the calculated value	No reduction	Complies
B''	<100 CFU of <i>B. subtilis</i> without phages	94	100	The mean number of CFU must not differ more than 50% between plate A'' and C''	% difference in the number of <i>B. subtilis</i> : 1%	Complies

Suitability of the counting method in the presence of LBP

[0138] A homogeneous suspension of the LBP was prepared. Two grams of the NCIMB 42488 sample (DS) were suspended in 18 mL CASO broth (10^{-1} dilution). A control with no test material included was prepared in the same way. The LBP suspension was further diluted 1:10 and 1:100 so dilutions 10^{-2} , and 10^{-3} of the LBP were created.

[0139] The test strains were prepared as cryo stocks with 43% glycerol and are stored at ≤ -64 °C. The cell counts were determined before. Dilutions in peptone water prepared to obtain <100 CFUs in 10 μ L as indicated in the table below.

Table 22. Preparation of test strains

Strain	MM strain No.	PRID	PCH	Given cell number in the cryostock [cfu/ mL]	Dilution in buffered peptone water	Calculated number of cfu/ 10 μ L
<i>S. aureus</i>	1178	695	6335	2.6×10^6	1 :270	96
<i>P. aeruginosa</i>	242	484	6131	3.1×10^5	1 :32	96
<i>B. subtilis</i>	241	486	6336	1.6×10^5	1 :17	94

[0140] Phage cocktail was prepared as described before. Tests were conducted as follows:

(D) Test without phages: To 1 ml aliquots of 10^{-1} , 10^{-2} , 10^{-3} LBP dilutions <100 CFU of *S. aureus* (in 10 μ l) were added. The entire volume was spread-plated on 3 CASO agar plates for each DS dilution. The test was performed in duplicate. *P. aeruginosa* and *B. subtilis* were tested in the same way.

(E) Test with phages: To 1 ml aliquots of 10^{-1} , 10^{-2} , 10^{-3} LBP dilutions <100 CFU of *S. aureus* (in 10 μ l) and 10 μ l phage cocktail were added and mixed shortly. The entire volume was spread-plated on 3 CASO agar plates for each DS dilution. The test was performed in duplicate. *P. aeruginosa* and *B. subtilis* were tested in the same way.

(F) Test without phages are without LBP: To 1 mL of the buffered peptone water <100 CFU of *S. aureus* (in 10 μ l) were added. The entire volume was spread-plated on 3 CASO agar plates. The test was performed in duplicate. *P. aeruginosa* and *B. subtilis* were tested in the same way.

(G) Test with phages and without LBP: To 1 mL of the buffered peptone water <100 CFU of *S. aureus* (in 10 μ l) and 10 μ l phage cocktail were added and mixed shortly. The entire volume was spread-plated on 3 CASO agar plates. The test was performed in duplicate. *P. aeruginosa* and *B. subtilis* were tested in the same way.

[0141] The plates D, E, F, and G were incubated for 3 days at 32.5 °C.

[0142] The surface-spread method was chosen for the enumeration of contaminating micro-organisms. Plate-counting was performed in duplicate for each test strain/ DS dilution combination. The arithmetic mean was taken of the counts per medium and the number of CFU in the original inoculum was calculated. When verifying the suitability of the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control in the absence of the LBP must be obtained.

Table 23. Recovery of *S. aureus* in the presence of the LBP- results

Suitability of the counting method in the presence of the LBP - 10^{-1} dilution (0.1g) – <i>S. aureus</i>						
Test	Description	Amount of DS [g], [CFU]	Number of <i>S. aureus</i> CFUs added	Volume of phage cocktail added	Recovered number of <i>S. aureus</i> CFU	Mean number of recovered <i>S. aureus</i> CFU
D	Test without phages	0,1 g, $1,1 \times 10^{11}$ CFU	96	0 μ l	0	-
					0	
E	Test with phages	0,1 g, $1,1 \times 10^{11}$ CFU	96	10 μ l	0	-
					0	
F	Control without LBP and without phages	0	96	0 μ l	47	50
					53	

G	Control without LBP and with phages	0	96	10 µl	82	89
					96	
Suitability of the counting method in the presence of the LBP -10 ⁻² dilution (0.01g) – <i>S. aureus</i>						
D'	Test without phages	0,01 g, 1.1 x 10 ¹⁰ CFU	96	0 µl	1	0.5
					0	
E'	Test with phages	0,01 g, 1.1 x 10 ¹⁰ CFU	96	10 µl	0	-
					0	
Suitability of the counting method in the presence of the LBP -10 ⁻³ dilution (0.001g) – <i>S. aureus</i>						
D''	Test without phages	0,001 g, 1.1 x 10 ⁹ CFU	96	0 µl	1	3.5
					6	
E''	Test with phages	0,001 g, 1.1 x 10 ⁹ CFU	96	10 µl	43	40
					37	

Table 24. Recovery of *P. aeruginosa* in the presence of the LBP- results

Suitability of the counting method in the presence of the LBP -10 ⁻¹ dilution (0.1g) – <i>P. aeruginosa</i>						
Test	Description	Amount of DS [g], [CFU]	Number of <i>P. aeruginosa</i> CFUs added	Volume of phage cocktail added	Recovered number of <i>P. aeruginosa</i> CFU	Mean number of recovered <i>P. aeruginosa</i> CFU
D	Test without phages	0,1 g, 1,1 x 10 ¹¹ CFU	96	0 µl	0	-
					0	
E	Test with phages	0,1 g, 1,1 x 10 ¹¹ CFU	96	10 µl	0	-
					0	
F	Control without LBP and without phages	0	96	0 µl	61	54.5
					48	
G	Control without LBP and with phages	0	96	10 µl	79	78
					77	
Suitability of the counting method in the presence of the LBP -10 ⁻² dilution (0.01g) – <i>P. aeruginosa</i>						
D'	Test without phages	0,01 g, 1.1 x 10 ¹⁰ CFU	96	0 µl	0	-
					0	
E'	Test with phages	0,01 g, 1.1 x 10 ¹⁰ CFU	96	10 µl	22	15
					8	
Suitability of the counting method in the presence of the LBP -10 ⁻³ dilution (0.001g) – <i>P. aeruginosa</i>						
D''	Test without phages	0,001 g, 1.1 x 10 ⁹ CFU	96	0 µl	20	16
					12	
E''	Test with phages	0,001 g, 1.1 x 10 ⁹ CFU	96	10 µl	24	25
					26	

Table 25. Recovery of *B. subtilis* in the presence of the LBP- results

Suitability of the counting method in the presence of the LBP -10 ⁻¹ dilution (0.1g) – <i>B. subtilis</i>						
Test	Description	Amount of DS [g], [CFU]	Number of <i>B. subtilis</i> CFUs added	Volume of phage cocktail added	Recovered number of <i>B. subtilis</i> CFU	Mean number of recovered <i>B. subtilis</i> CFU
D	Test without phages	0,1 g, 1,1 x 10 ¹¹ CFU	94	0 µl	6	4
					2	
E	Test with phages	0,1 g, 1,1 x 10 ¹¹ CFU	94	10 µl	0	-
					0	
F	Control without LBP and without phages	0	94	0 µl	72	81.5
					91	
G	Control without LBP and with phages	0	94	10 µl	110	102
					94	
Suitability of the counting method in the presence of the LBP -10 ⁻² dilution (0.01g) – <i>B. subtilis</i>						
D'	Test without phages	0,01 g, 1.1 x 10 ¹⁰ CFU	94	0 µl	54	56.5
					59	
E'	Test with phages	0,01 g, 1.1 x 10 ¹⁰ CFU	94	10 µl	97	90
					83	
Suitability of the counting method in the presence of the LBP -10 ⁻³ dilution (0.001g) – <i>B. subtilis</i>						
D''	Test without phages	0,001 g, 1.1 x 10 ⁹ CFU	94	0 µl	82	80
					78	
E''	Test with phages	0,001 g, 1.1 x 10 ⁹ CFU	94	10 µl	84	82.5
					81	

[0143] The number of test microorganisms recovered from the LBP sample (plates D) was compared to the number of test microorganisms recovered from the control preparation (plates F). If the growth is inhibited (reduction by a factor greater than 2) the decision tree shown in Ph. Eur. figure 2.6.36.1 should be followed.

Table 26. Inhibitory activity without application of phages- results

Test micro-organism	<i>S. aureus</i>			<i>P. aeruginosa</i>			<i>B. subtilis</i>		
	0.1 g	0.01 g	0.001 g	0.1 g	0.01 g	0.001 g	0.1 g	0.01 g	0.001 g
No. of recovered test micro-organisms from the control without LBP (F)	50			54.5			81.5		
No. of recovered test micro-	0	0.5	3.5	0	0	16	4	56.5	80

organism from the LBP (D)									
Reduction factor [D/F]	0	0.01	0.07	0	0	0.29	0.05	0.69	0.98
Acceptance criterion	$0.5 \leq [D/F] \leq 2$								
Evaluation	Failure	Failure	Failure	Failure	Failure	Failure	Failure	Complies	Complies

[0144] The number of test microorganisms recovered from the LBP sample (plates E) will be compared to the number of test microorganisms recovered from the control preparation (plates G). If the growth is inhibited (reduction by a factor greater than 2) the decision tree shown in Ph. Eur. figure 2.6.36.1 should be followed.

Table 27. Inhibitory activity with application of phages- results

Test micro-organism	<i>S. aureus</i>			<i>P. aeruginosa</i>			<i>B. subtilis</i>		
Amount of LBP	0.1 g	0.01 g	0.001 g	0.1 g	0.01 g	0.001 g	0.1 g	0.01 g	0.001 g
No. of recovered test micro-organisms from the control without LBP (G)	89			78			102		
No. of recovered test micro-organism from the LBP (E)	0	0	40	0	15	25	0	90	82.5
Reduction factor [E/G]	0	0	0.45	0	0.19	0.32	0	0.88	0.81
Acceptance criterion	$0.5 \leq [E/G] \leq 2$								
Evaluation	Failure	Failure	Failure	Failure	Failure	Failure	Failure	Complies	Complies

Test for specified microorganisms

[0145] A homogeneous suspension of the LBP was prepared. Seven grams of the NCIMB 42488 sample (DS) were suspended in 63 mL buffered peptone water (10^{-1} dilution). A control with no test material included was prepared in the same way. The LBP suspension was further diluted 1:10 and 1:100 so dilutions 10^{-2} , and 10^{-3} of the LBP were created.

[0146] The test strains were prepared as cryo stocks with 43% glycerol and are stored at ≤ -64 °C. The counts were determined before. Dilutions in peptone water were prepared to obtain <100 CFUs in 100 μ l (low number of specified micro-organisms) as indicated in the table below. Additionally, undiluted suspensions of the test strains were used to evaluate the overall feasibility of the detection procedure.

Table 28. Preparation of test strains

Strain	MM strain No.	PRID	PCH	Given cell number in the cryostock [cfu/ mL]	Dilution in buffered peptone water	Calculated number of cfu/ 100 μ L
<i>S. aureus</i>	1178	695	6335	2.6×10^6	1:2700	96
<i>S. aureus</i>	1718	695	6335	2.6×10^6	undiluted	2.6×10^5
<i>B. cereus</i>	519	704	6360	3.8×10^5	1:390	97
<i>B. cereus</i>	519	704	6360	3.8×10^5	undiluted	3.8×10^4

[0147] The phage cocktail was prepared as described above.

[0148] Test strains suspensions with the counts of <100 CFU in the volume of 100 μ l each were spread-plated on CASO agar plates covered or not with phage cocktail. The plates were incubated for <18 h at 32.5 °C and the counts of micro-organisms were determined. To prepare the plates covered with phages, 100 μ l of the phage cocktail was spread-plated on the agar surface and dried shortly before use.

Table 29. Test for growth promoting properties of solid media- results

		A - Calculated before no. of CFU / plate	B - No. of CFU/ plate found	Test strain recovery (B/A)	Acceptance criteria	Evaluation
H – Solid media without phages	<i>S. aureus</i> (< 100 cfu)	96	96	100 %	Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium and does not differ by a factor greater than 2	Complies
	<i>B. cereus</i> (< 100 cfu)	97	111	114 %		Complies
I – Solid media with phages	<i>S. aureus</i> (< 100 cfu)	96	82	85 %		Complies
	<i>B. cereus</i> (< 100 cfu)	97	106	109 %		Complies

[0149] The test for inhibitory properties of solid media was not applicable. The solid media used in this study are not selective for cultivation of *S. aureus* and *B. cereus*, but are selectively inhibiting the growth of LBP micro-organism.

[0150] The pre-enrichment of the target micro-organisms in the presence of the LBP was conducted in CASO broth supplemented with phage cocktail that should neutralize the inhibitory activity of the LBP.

[0151] To 90 mL CASO broth 10 mL of the test sample in three different dilutions, 100 µl of the test strains preparations, and 100 µl of phage cocktail were added. The mixes were incubated for 18 h at 32.5 °C without agitation. Positive controls for detection of *S. aureus* and *B. cereus* were prepared by adding 10 ml of buffered peptone water in place of LBP to 90 mL CASO broth, inoculation with 100 µl of test strain suspension < 100 CFU each, and 100 µl phage cocktail each. Negative controls were prepared by inoculation of 90 mL CASO broth with 10 mL of the 10^{-1} , 10^{-2} , 10^{-3} dilutions of the test sample and 100 µl phage cocktail. No *S. aureus* or *B. cereus* were added.

[0152] A reference sample with the microbial composition only was produced by inoculation of 90 mL CASO broth with 10 mL of the 10^{-1} , 10^{-2} , 10^{-3} dilutions of the test sample.

[0153] All cultures were incubated for 18 h at 32.5 °C without agitation.

[0154] From each culture prepared as described previously two 10 µl dilution streaks on CASO agar were made. The plates were incubated at 32.5 °C for 18 h.

[0155] To prepare the plates covered with phages, 100 µl of the phage cocktail was spread-plated on the agar surface and dried shortly before use. Dilution streaks from each culture prepared as described previously were made on CASO agar plates covered with phages.

[0156] The plates were incubated at 32.5 °C for 18 h.

[0157] Whenever *S. aureus* colonies were observed in the presence of the LBP typical colonies, at least 2 separated colonies that were visually identified as *S. aureus* were picked in order to confirm their identity using Staphaurex™ latex agglutination test. The test was conducted according to the manufacturer description.

[0158] Whenever *B. cereus* colonies were observed in the presence of the LBP typical colonies, at least 2 separated colonies that were visually identified as *B. cereus* were picked in order to confirm their identity via microscopy (Gram-positive rods, endospores forming).

[0159] Buffered peptone water, CASO broth and phage cocktail were spread-plated each in the volume of 100 µl on CASO agar. To test the processing environment for sterility, one CASO agar plate

was placed under the laminar flow bench and was kept open while processing the samples. The plates were incubated for 3 days at 32.5 °C.

[0160] The results from the test for suitability of the test method for detection of specified micro-organisms are described in the table below.

Table 30. Test for specified micro-organisms- results

Test for specified microorganisms – suitability of the test method							
Test	Dilution of the DS	Phages in the pre-enrichment	Phages in subculture	Calculated number of <i>S. aureus</i> in the pre-enrichment	Calculated number of <i>B. cereus</i> in the pre-enrichment	Detection of test microorganism	
						Repl. 1	Repl. 2
Subculture without phages	10 ⁻¹	+	-	96	-	-	-
	10 ⁻²	+	-	96	-	-	-
	10 ⁻³	+	-	96	-	+	+
	10 ⁻¹	+	-	2,6*10 ⁵	-	-	-
	10 ⁻²	+	-	2,6*10 ⁵	-	-	-
	10 ⁻³	+	-	2,6*10 ⁵	-	+	+
	10 ⁻¹	+	-	-	97	-	-
	10 ⁻²	+	-	-	97	-	-
	10 ⁻³	+	-	-	97	+	+
	10 ⁻¹	+	-	-	3,8x10 ⁴	-	-
	10 ⁻²	+	-	-	3,8x10 ⁴	-	+
	10 ⁻³	+	-	-	3,8x10 ⁴	+	+
Subculture with phages	10 ⁻¹	+	+	96	-	-	-
	10 ⁻²	+	+	96	-	-	-
	10 ⁻³	+	+	96	-	+	+
	10 ⁻¹	+	+	2,6*10 ⁵	-	-	+
	10 ⁻²	+	+	2,6*10 ⁵	-	+	+
	10 ⁻³	+	+	2,6*10 ⁵	-	+	+
	10 ⁻¹	+	+	-	97	-	-
	10 ⁻²	+	+	-	97	-	-
	10 ⁻³	+	+	-	97	+	+
	10 ⁻¹	+	+	-	3,8x10 ⁴	-	-
	10 ⁻²	+	+	-	3,8x10 ⁴	-	+
	10 ⁻³	+	+	-	3,8x10 ⁴	+	+

Table 31. Controls in the test for specified micro-organisms – subcultures on the plates without phages

Subculture without phages, controls									
Control	Description (pre-enrichment)	Calculated number of test micro-organism	Detection of test micro-organism		Confirmation of identity		Acceptance criteria	Results	Evaluation
			Repl. 1	Repl. 2	Repl. 1	Repl. 2			
Positive control for <i>S. aureus</i> (no DS)	<100 CFU <i>S. aureus</i> + phages	96	+	+	+	+	growth and detection of <i>S. aureus</i>	<i>S. aureus</i> detected	Complies
Positive control for <i>B. cereus</i> (no DS)	<100 CFU <i>B. cereus</i> + phages	97	+	+	+	+	growth and detection of <i>B. cereus</i>	<i>B. cereus</i> detected	Complies
Negative control (No test micro-organism)	DS 10 ⁻¹ + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	growth of <i>E. gallinarum</i> only	Complies
Negative control (No test micro-organism)	DS 10 ⁻² + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	growth of <i>E. gallinarum</i> only	Complies
Negative control (No test micro-organism)	DS 10 ⁻³ + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	Limited growth of <i>E. gallinarum</i> only	Complies
reference sample with DS only	DS 10 ⁻¹	0	n.a.	n.a.	n.a.	n.a.	growth of <i>E. gallinarum</i> only	Growth of <i>E. gallinarum</i> only	Complies
reference sample with DS only	DS 10 ⁻²	0	n.a.	n.a.	n.a.	n.a.	growth of <i>E. gallinarum</i> only	Growth of <i>E. gallinarum</i> only	Complies
reference sample with DS only	DS 10 ⁻³	0	n.a.	n.a.	n.a.	n.a.	growth of <i>E. gallinarum</i> only	Growth of <i>E. gallinarum</i> only	Complies

Table 32. Controls in the test for specified micro-organisms – subcultures on the plates with phages

Subculture with phages, controls									
Control	Description (pre-enrichment)	Calculated number of test micro-organism	Detection of test micro-organism		Confirmation of identity		Acceptance criteria	Results	Evaluation
			Repl. 1	Repl. 2	Repl. 1	Repl. 2			
Positive control for <i>S. aureus</i> (no DS)	<100 CFU <i>S. aureus</i> + phages	96	+	+	+	+	growth and detection of <i>S. aureus</i>	<i>S. aureus</i> detected	Complies
Positive control for <i>B. cereus</i> (no DS)	<100 CFU <i>B. cereus</i> + phages	97	+	+	+	+	growth and detection of <i>B. cereus</i>	<i>B. cereus</i> detected	Complies
Negative control (No test micro-organism)	DS 10 ⁻¹ + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	No growth of <i>E. gallinarum</i>	Complies
Negative control (No test micro-organism)	DS 10 ⁻² + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	No growth of <i>E. gallinarum</i>	Complies
Negative control (No test micro-organism)	DS 10 ⁻³ + phages	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	Only few <i>E. gallinarum</i> colonies observed	Complies
reference sample with DS only	DS 10 ⁻¹	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	Only few <i>E. gallinarum</i> colonies observed	Complies
reference sample with DS only	DS 10 ⁻²	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	Only few <i>E. gallinarum</i> colonies observed	Complies
reference sample with DS only	DS 10 ⁻³	0	n.a.	n.a.	n.a.	n.a.	no growth or growth of <i>E. gallinarum</i> only	No growth of <i>E. gallinarum</i>	Complies

Table 33. Sterility controls- results

Plate(s)	Number of CFU	Acceptance criteria	Result	Evaluation
Peptone water plated on CASO agar	0	No microbial growth	No microbial growth	Complies
CASO broth plated on CASO agar	0	No microbial growth	No microbial growth	Complies
100 µl phage cocktail on CASO Agar	0	No microbial growth	No microbial growth	Complies
Environmental control	0	No microbial growth	No microbial growth	Complies

[0161] The described method is suitable for the enumeration of *B. subtilis* provided that DS is diluted at least 1:100, i.e. the *B. subtilis* can be reliably enumerated in 0.01 g of DS. For the enumeration of *B. subtilis* no significant improvement was observed by the use of phage cocktail as an agent neutralising the inhibitory activity of the LBP.

[0162] The method is not suitable for the enumeration of *S. aureus* and *P. aeruginosa*, however the use of phage cocktail and dilution of the DS significantly improved the recovery of these test micro-organisms in the presence of the LBP.

[0163] The method is suitable for the detection of *S. aureus* and *B. cereus* in the presence of the LBP provided that the DS is diluted at least 1:1000, i.e. low numbers of deliberately added test micro-organisms can be reliably detected in 0.01 g or lower amount of the DS.

[0164] The above experiments show that it is possible to use the process of the present invention to determine whether a microbial composition containing a therapeutic bacterial population contains any unwanted organisms. The principles shown by these experiments can be applied to other microbial compositions for therapeutic use, as will be appreciated by a person skilled in the art.

CLAIMS

1. A process for analysing a composition comprising therapeutic bacteria, wherein the process comprises:
 - (a) culturing the composition under conditions which selectively inhibit the growth of the therapeutic bacteria; and
 - (b) analysing the cultured composition of step (a) to determine whether an unwanted organism was present in the composition.
2. The process of claim 1, wherein the growth inhibition of the therapeutic bacteria in step (a) is at least two fold, at least three fold, at least four fold, at least five fold, at least ten fold, at least twenty fold, at least fifty fold than the growth inhibition of the unwanted organism.
3. The process of any one of claims 1 to 2, wherein, when step (b) determines that at least one unwanted organism was present in the microbial composition, the process includes the step of identifying the or each unwanted organism.
4. The process of claim 3, wherein the microbial composition is analysed to determine whether any unwanted organisms is a virus, archaea, eukaryotic cell or an anaerobic or aerobic bacterium.
5. The process of claim 5, wherein the unwanted organism is a bacterium from the *Clostridia* or *Salmonella* genera or the *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Escherichia coli* species, or the like or a mixture of such unwanted organisms.
6. The process of any one of claims 1 to 5, wherein the unwanted organism is a cellular organism and the ratio of the number of cells of the therapeutic bacteria to the number of cells of the unwanted organism(s) in the microbial composition prior to the culturing step is $\geq 1,000 : 1$, $\geq 10,000 : 1$, $\geq 100,000 : 1$, $\geq 1,000,000 : 1$, $\geq 10,000,000 : 1$, $\geq 100,000,000 : 1$, $\geq 1,000,000,000 : 1$ or $\geq 1,10,000,000,000 : 1$.
7. The process of any one of claims 1 to 6, wherein the growth of the therapeutic bacteria is selectively inhibited by selecting the pH, temperature and/or ionic strength of the culture medium.

8. The process of any one of claims 1 to 6, wherein the growth of the therapeutic bacteria is selectively inhibited by addition to the culture medium of an agent which inhibits the growth of the therapeutic bacteria.
9. The process of claim 8, wherein the agent reduces or prevents the therapeutic bacteria from multiplying.
10. The process of claim 8 or claim 9, wherein the agent actively kills at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9% or 100% of the therapeutic bacteria present in the composition.
11. The process of any one of claims 8 to 10, wherein the agent is a bacteriophage, lysozyme or antibiotic.
12. The process of claim 11, wherein the agent is not an antibiotic.
13. The process of claim 11, wherein the agent is a bacteriophage.
14. The process of claim 13, wherein the bacteriophage selectively inhibits one or more bacteria of the genus *Enterococcus*.
15. The process of claim 13 or 14, wherein the agent is a bacteriophage having a DNA genome comprising SEQ ID NO: 1 or a functional equivalent thereof.
16. The process of claim 13 or 14, wherein the agent is a bacteriophage having a DNA genome comprising SEQ ID NO: 2 or a functional equivalent thereof.
17. The process of claim 15 or claim 16, wherein the functional equivalent is a bacteriophage comprising a DNA genome having at least 70%, at least 80%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at

least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2.

18. The process of claim 16 or 17, wherein the bacteriophage is NCIMB 43666.

19. The process of claim 15 or 17, wherein the bacteriophage is NCIMB 43667.

20. The process of any one of claims 1 to 19, wherein the therapeutic bacteria comprise or consist of anaerobic bacteria, obligate anaerobic bacteria, facultative anaerobic bacteria and / or microaerophilic bacteria.

21. The process of claim 20, wherein the microbial composition comprises one or more bacteria from the following genera: *Enterococcus* (e.g. *Enterococcus gallinarum*, *Enterococcus caselliflavus*, *Enterococcus faecalis* or *Enterococcus faecium*), *Blautia* (e.g. *Blautia hydrogenotrophica*, *Blautia stercoris*, *Blautia wexlerae* or *Blautia producta*), *Bacteroides* (e.g. *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides vulgatus*, *Bacteroides dorei* or *Bacteroides copricola*), *Faecalibacterium* (e.g. *Faecalibacterium prausnitzii*), *Bariatricus* (e.g. *Bariatricus massiliensis*), *Bifidobacterium* (e.g. *Bifidobacterium breve*, *Bifidobacterium adolescentis* or *Bifidobacterium longum*), *Roseburia* (e.g. *Roseburia hominis*, *Roseburia intestinalis* or *Roseburia inulinivorans*), *Flavonifractor* (e.g. *Flavonifractor plautii*), *Anaerotruncus* (e.g. *Anaerotruncus colihominis*), *Parabacteroides* (e.g. *Parabacteroides distasonis*, *Parabacteroides goldsteinii*, *Parabacteroides merdae* or *Parabacteroides johnsonii*), *Erysipelatoclostridium* (e.g. *Erysipelatoclostridium ramosum*), *Megasphaera* (e.g. *Megasphaera massiliensis*), *Pediococcus* (e.g. *Pediococcus acidilacticii*), *Eubacterium* (e.g. *Eubacterium contortum*, *Eubacterium fissicatena*, *Eubacterium eligens*, *Eubacterium hadrum*, *Eubacterium hallii* or *Eubacterium rectale*), *Ruminococcus* (e.g. *Ruminococcus torques*, *Ruminococcus gnavus* or *Ruminococcus bromii*), *Pseudoflavonifractor* (e.g. *Pseudoflavonifractor capillosus*), *Clostridium* (e.g. *Clostridium nexile*, *Clostridium hylemonae*, *Clostridium butyricum*, *Clostridium tertium*, *Clostridium disporicum*, *Clostridium bifermentans*, *Clostridium inocuum*, *Clostridium mayombeii*, *Clostridium bolteae*, *Clostridium bartlettii*, *Clostridium symbiosum* or *Clostridium orbiscindens*), *Coprococcus* (e.g. *Coprococcus comes* or *Coprococcus catus*), *Acetivibrio* (e.g. *Acetivibrio ethanoligignens*), *Dorea* (e.g. *Dorea longicatena*) or any genera of the family *Lachnospiraceae*.

22. The process of claim 4 or any claim dependent thereon wherein the determination of the presence of an unwanted organism in the microbial composition comprises the identification of cellular products in the cultured microbial composition, preferably cellular products not produced by the therapeutic bacteria and / or which are indicative of the presence of specific pathogenic bacteria such as organisms from the *Clostridia* and *Salmonella* genera and the *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* species.
23. The process of any one of claims 1-21, wherein step (b) comprises GC-MS or MALDI-TOF-MS analysis.
24. The process of any one of claims 1 to 23, wherein the microbial composition at the start of culturing step a) comprises 1×10^{12} CFU or less of therapeutic bacteria.
25. The process of any one of claims 1 to 23, wherein the microbial composition at the start of culturing step a) comprises 5×10^{11} CFU or less of therapeutic bacteria.
26. The process of any one of claims 1 to 23, wherein the microbial composition at the start of culturing step a) comprises 1×10^{11} CFU or less of therapeutic bacteria.
27. The process of any one of claims 1 to 26, wherein the microbial composition at the start of culturing step a) comprises one or more excipients, for example, lyoprotectants, preservatives, antioxidants, stabilisers, prebiotic compounds, pharmaceutically acceptable carriers or diluents, binders, lubricants, suspending agents, coating agents, solubilising agents, or the like.
28. A kit comprising an agent which selectively inhibits a first bacterial population and instructions to use the agent in the process of any one of claims 1 to 27.
29. A bacteriophage having a DNA genome comprising SEQ ID NO: 1 or SEQ ID NO: 2, or a functional equivalent thereof.
30. A bacteriophage having a DNA genome having at least 70%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at

least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2.

31. A composition comprising one or more bacteriophages, wherein the composition selectively inhibits the growth of one or more therapeutic bacteria in a microbial composition.

32. A composition according to claim 31, further comprising a stabiliser, preservative and / or additive.

33. A composition according to claim 31 or 32, wherein the composition comprises a bacteriophage according to claim 28 or 29.

34. A composition according to claim 33, wherein the composition comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 bacteriophages.

35. A composition suitable for use in the process of any one of claims 1 to 27, comprising two or more bacteriophages, wherein the composition selectively inhibits the growth of one or more therapeutic bacteria in the microbial composition.

36. A method of preparing a pharmaceutical composition comprising the steps of

- (a) culturing therapeutic bacteria;
- (b) performing the process of any one of claims 1-27 on at least a fraction of the culture of step (a); and
- (c) formulating a pharmaceutical composition from the therapeutic bacteria of step (a).