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(54) **Title:** BIOFILM CONTROL USING *BACILLUS SUBTILIS* AND *VACCINIUM MACROCARPON* PROANTHOCYANIDINS

(57) **Abstract:** A biocide composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH.



**BIOFILM CONTROL USING *BACILLUS SUBTILIS* AND
VACCINIUM MACROCARPON PROANTHOCYANIDINS**

[0001] CROSS REFERENCE TO RELATED APPLICATION

[0002] This application claims the benefit of U.S. Provisional Application Serial No. 62/168,993, filed June 1, 2015, the disclosure content of which is hereby incorporated by reference in its entirety.

[0003] FIELD OF THE INVENTION

[0004] The Invention relates to the control of pathogenic microorganisms by altering the ecology of sessile biofilms in food production and healthcare settings.

[0005] BACKGROUND OF THE INVENTION

[0006] Biofilms are common in nature and are often associated with unwanted biofouling. Problems caused by biofilms include the ability of *Legionella* to persist in cooling towers and other water systems due to its complex interrelationships with other microflora (Murga et al., 2001), fouling of oil and gas pipelines and ships' hulls, dental plaque causing tooth decay and microbial colonisation of indwelling or percutaneous medical devices such as catheters, artificial valves, joints, stents etc.

[0007] Classical microbiological studies have concentrated on bacteria in their planktonic state – single organisms or small groups suspended in aqueous culture – but most bacteria in nature colonise natural and man-made surfaces using exogenous polysaccharides (the glycocalyx) for adhesion, then multiply to form colonies which grow into complex, sessile biofilms (Costerton et al., 1995).

[0008] Biofilms are complex, irregular, heterogeneous hydrated structures where a consortium of microcolonies of bacteria can divide freely (Lewandowski, 2000). Inhabiting a biofilm confers significant advantages on its resident bacteria. They are protected from environmental threats such as desiccation, antibiotics (Ishida et al., 1998) and biocides

(McDonnell and Russell, 1999), and form symbiotic relationships with other residents allowing exchange of nutrients, metabolites and genetic material.

[0009] The glycocalyx is the principal component of the extracellular matrix (ECM) – which provides the biofilm’s structural integrity and is chemically similar to the bacterial capsule surrounding pathogens such as *Streptococcus*, *Klebsiella*, *Pseudomonas* and *Neisseria*. The ECM is more diffuse and less distinct than a capsule and accounts for 50-90% of the organic carbon content of the biofilm (Flemming, Wingender and Mayer, 2000). It has been shown that *B. subtilis* biofilms are more nonwetting (that is, they have greater repellency to water) than Teflon® and are impervious even to 80% ethanol (Epstein et al., 2011), hence biofilms’ recalcitrance.

[0010] The impact of biofilms on public health is well documented from diseases such as cystic fibrosis and otitis media to *Legionella*, mycobacteria and *Helicobacter* in potable water systems to colonisation of medical devices. Their nonwetting properties and recalcitrance suggests new paradigms are needed to control them. One example is to disrupt the quorum sensing that bacteria use to communicate with each other (Bauer and Robinson, 2002). The compositions described herein, uses of said compositions, and methods of applying and using the same provide for a novel approach towards biofilm control.

[0011] SUMMARY OF THE INVENTION

[0012] In a preferred embodiment, the invention is directed to a biocide composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH.

[00013] A further embodiment is directed to a biocide composition comprising an effective amount of *Bacillus subtilis* and *Vaccinium macrocarpon* proanthocyanidins. In a further embodiment, the composition further comprising a suitable surfactant. In a further embodiment, the composition further comprising an opacifier, a buffer, and a fragrance. In a further embodiment, the composition wherein the suitable surfactant is a non-ionic and amphoteric surfactant. In a further embodiment, the composition comprising two or more surfactants. In a further embodiment, the composition further comprising a xanthan-sorbate carrier. In a further embodiment, the composition further comprising a surface tension decreasing agent. In a further embodiment, the composition wherein the effective amount of the *Bacillus subtilis* is 1×10^8 cfu/ml. In a further embodiment, the composition further comprising an effective amount of *Bacillus licheniformis*.

[00014] In a further embodiment, the use of the composition above to control environmental pathogenic bacteria. In a further embodiment, the composition further comprising natural antibiotics produced systemically from the applied composition. In a further embodiment, wherein the composition further comprising lantibiotics. In a further embodiment, the use of the composition to digest organic soils. In a further embodiment, the use of the composition to disrupt biofilm formation. In a further embodiment, the use of the composition to mediate biocidal activity.

[00015] In a further embodiment, a composition suitable for artificially generating a benign biofilm, wherein said composition comprises an effective amount of *Bacillus subtilis*, *Vaccinium macrocarpon* proanthocyanidins, a suitable carrier, and a surfactant. In a further embodiment, wherein the suitable carrier is a xanthan-sorbate carrier. In a further embodiment, use of the composition to generate a benign biofilm.

[00016] In a further embodiment, a method of disrupting an existing biofilm comprising:

- 1) applying a composition comprising an effective amount of *Bacillus subtilis*, *Vaccinium macrocarpon* proanthocyanidins, a suitable carrier, and a surfactant to surface for a sufficient time to transfer the *Vaccinium macrocarpon* proanthocyanidins to said existing biofilm, wherein said *Vaccinium macrocarpon* proanthocyanidins disrupt said biofilm surface by reducing the mobility of existing bacteria; and
- 2) generating a benign biofilm surface from colonized *Bacillus subtilis* from said application.

[00017] In a further embodiment, wherein the method utilizes a suitable carrier, which is a xanthan-sorbate carrier.

[00018] In a further embodiment, a biocide composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH.

[00019] A further embodiment is directed to a biofilm control composition comprising one or more strains of *Bacillus subtilis*, which are prodigious producers of natural lantibiotics and other biocidal moieties, acting synergistically with a suite of biologically-active extracts; wherein, in combination with suitable surfactant and A. proanthocyanidin components, said natural lantibiotics and biocidal moieties provide a means for controlling biofilms in food production and healthcare by – *inter alia* - excluding pathogens as well as direct and indirect biocidal activity.

[00020] A further embodiment is directed to a method for reducing the occurrence of gram-negative bacteria on a surface comprising:

- 1) applying to a surface, a composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH; and
- 2) mechanically removing the composition from the surface to remove contaminants removed by the composition.

[00021] A further embodiment is directed to a method of reducing a pathogenic biofilm on a surface comprising:

- 1) applying a composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH to a surface;
- 2) forming colonies of said bacteria from said composition on said surface so as to out compete the pathogenic bacteria on said surface;
- 3) degrading the chemical bonds on the pathogenic bacteria biofilm with the proanthocyanidines; and
- 4) reducing the numbers of pathogenic bacteria through quorum sensing.

[00022] In a further embodiment, use of any one of the methods or compositions for reducing the number of colony forming units of gram-negative bacteria on a surface.

[00023] Description of the Invention

[00024] The National Institutes of Health (NIH) has estimated biofilms are involved in 80% of all infections (PA-03-047, 2002).

[00025] Disease-causing microbes (including nosocomial pathogens) use biofilms as an ideal habitat for reproduction and exchange of genetic material via plasmids.

[00026] It has now been shown that bacteria from different genera can exchange resistance plasmids, and this ability to cross the species barrier has led to the rapid global spread of many different bacteria becoming resistant to both common antibiotics and antibiotics of ‘last resort’. This has already been found in healthcare facilities in the USA.

[00027] In addition, biofilms are impervious to traditional chemical biocides and antibiotics (Epstein et al., 2011) and so cannot be controlled by conventional biocidal chemicals.

[00028] Biofilms in nature often contain a variety of bacteria, algae and protozoa but it is possible to grow a biofilm with a single species. The *Bacillus subtilis* in the Invention can readily switch from its motile, planktonic state to grow in bundled chains and then multicellular communities (Kearns et al., 2005). This ability to form complex structures gives *B. subtilis* the ability to form extremely robust biofilms and its ability to exclude other organisms combined with proanthocyanidins’ ability to disrupt existing biofilms makes the Invention a novel means to re-engineer existing biofilm ecology

[00029] The impact of bacterial biofilms in public health is well documented. They are implicated in a considerable proportion of nosocomial infections where their presence can cause mortality or significant morbidity. Their resident organisms also produce foodborne diseases affecting 1 in 6 Americans (or 48 million people) annually causing 128,000 hospitalizations and 3,000 deaths. Overuse of antibiotics in animal feed is breeding resistance adding to the issue of pan-resistant organisms. Therefore, control and modification of the

biofilm provides an opportunity to disrupt existing biofilms and to replace them with an innocuous biofilm.

[00030] The invention provides a means of modifying the microbial ecology of biofilms to exclude and destroy pathogens by replacing them with non-pathogenic bacteria. The invention uses a mix of bacteria that invade biofilms to competitively exclude and destroy pathogenic bacteria. These introduced bacteria produce natural antibiotics to kill pathogenic bacteria and biosurfactants to degrade organic soiling. Furthermore, the antibiotics the introduced bacteria produce are not suitable for use in treating human disease and so will not drive antibiotic resistance. This combination of biocidal activity against pathogenic bacteria and effective removal of soiling offers a new tool for hygiene and infection control not reliant on traditional means that merely drive bacterial resistance.

[00031] The *Bacillus subtilis* species used in the invention is not a human pathogen (Osipova et al., 1998). The species utilized is a Gram-positive spore former and a facultative anaerobe (Nakano and Zuber, 1998) and is particularly well suited to this application due to its lack of pathogenicity, ubiquity in the environment (principally soil) and ease of culture.

[00032] We have demonstrated with the invention that it is possible to apply suitable strains of *B. subtilis* in the same way as traditional cleaning products and artificially introduce a biofilm that digests organic soils by producing a suitable suite of exogenous enzymes. The Invention can be used in the same way as a traditional 'spray and wipe' cleaning product, namely, application to a surface or a cloth, and mechanically wiping the surface.

Furthermore, biofilms offer a new paradigm for hygiene and infection control; their ubiquity and recalcitrance is only now becoming apparent and the ability of the invention to re-engineer existing biofilms and digest body fluids, fats, dirt, grease and grime as well as excluding pathogenic bacteria presents an environmentally responsible and effective solution in both food hygiene and healthcare.

[00033] Accordingly, a biofilm control composition comprises the use of one or more strains of *Bacillus subtilis*, which are prodigious producers of natural lantibiotics and other biocidal moieties, acting synergistically with a suite of biologically-active extracts. Together, in combination with suitable surfactant and A. proanthcyanidin components, provides a means for controlling biofilms in food production and healthcare by – *inter alia* - excluding pathogens as well as direct and indirect biocidal activity.

[00034] The composition functions through several primary actions, including competitive exclusion, reduction of the native biofilm and quorum sensing, wherein present bacteria are converted from pathogenic to benign.

[00035] The competitive exclusion is generated as the bacteria provided in the composition are generally stronger, grow faster, and can out compete the pathogenic bacteria. Accordingly, application of these bacteria utilize the resources that pathogenic bacteria need to grow and flourish. As these resources are utilized and usurped by the benign bacteria, the pathogens are competitively excluded and begin to die out. Furthermore, compositions generated by the *Bacillus* strains, such as antibiotics, and other components, e.g. A. Proanthcyanidin, among others, also attack the pathogenic bacteria, ultimately leading to death of some of these bacteria. This results in a reduction in efficiency of the biofilm and reduces the spread and exchange of genetic materials between the pathogenic bacteria.

[00036] Indeed, the cranberry A. proanthcyanidin compounds are particularly suited in the compositions disclosed herein for the properties of disrupting of existing biofilms. Because of the natural resistance of bacteria within a biofilm to antibiotics and desiccation, effective control and eradication of the existing biofilm, requires some action to attack and disrupt the protective nature of the biofilm. Accordingly, the proanthcyanidin compounds provide a mechanism to attack the weakened biofilm to aid in its removal and breakdown.

[00037] Finally, as the pathogenic bacteria are outcompeted and are attacked by the compositions generated by the *Bacillus* strains, in some cases, some of the bacteria remaining are recruited and or courted based on quorum sensing and become part of the biofilm of the benign bacteria.

[00038] This combination, based on the studies disclosed herein, is completely effective against Gram-negative organisms where the unique nature of their cell wall makes them resistant to many classes of antibiotics including beta-lactams and carbapenems. Gram-negative organisms are also a principal cause of food poisoning and foodborne intoxication including *Campylobacter*, *E. coli* and *Salmonella*. They are also highly efficient at up-regulating or acquiring mechanisms of antibiotic drug resistance, especially in the presence of antibiotic selection pressure and so are a leading cause of preventable nosocomial infections.

[00039] The invention controls and renders harmless biofilms and can be used in healthcare and food production environments to competitively exclude clinically significant pathogens as well as producing enzymes to digest soils without the need for toxic chemicals. Based on our studies, the introduction of compositions as described herein into food production areas will decrease the requirement for antibiotics, for example, in animal feed, in addition to its ability to control pathogenic bacteria and biofilms, as well as removing soiling.

[00040] Therefore, a preferred embodiment of the composition comprises a blend of *Bacillus subtilis* strains which produce prodigious amounts of the natural biosurfactant surfactin as well as biocidal concentrations of subtilisin and lanthionine-containing peptide antibiotics, which competitively exclude pathogenic species. Furthermore, the *in situ* production of α -amylase enzymes by the Invention detach biofilm exopolysaccharide components; that these microbially-mediated effects on pathogens work synergistically with addition of North American cranberry (*Vaccinium macrocarpon*) proanthocyanidins to inhibit

biofilm development and that the amylases, proteases and lipases also produced *in situ* make the invention a highly effective and environmentally-responsible cleaning product.

[00041] A further embodiment is directed to a biocide composition comprising an effective amount of *Bacillus subtilis*, *Bacillus licheniformis* and *Vaccinium macrocarpon* proanthocyanidins, wherein the biocidal activity is mediated by natural enzymes and antibiotics formed *in situ*. Preferably, the in-use concentration of the *Bacillus* strains is about 1×10^8 cfu/ml. Further embodiments may also advantageously combine *Bacillus amyloliquefaciens* strains.

[00042] In a further embodiment environmental pathogenic bacteria are controlled using competitive exclusion through administration of the composition.

[00043] In a further embodiment, a composition wherein the environmental pathogenic bacterial are controlled using natural antibiotics including but not limited to lantibiotics, surfactin and subtilin and wherein lantibiotics are antagonistic to *Clostridium* spores (Andersen, 1952) and will be adsorbed onto dormant spores which will be destroyed as soon as they start the process of germination (Michener, 1955).

[00044] In a further embodiment, the composition digests organic soils due to its *in situ* production of amylases, proteases and lipases.

[00045] In a further embodiment the composition disrupts biofilm formation due to its production *in situ* of α -amylases.

[00046] In a further embodiment the composition disrupts biofilm formation due to inclusion of *V. macrocarpon* proanthocyanidins.

[00047] In a further embodiment, the composition further comprises a xanthan-sorbate carrier mix with non-ionic and amphoteric surfactants. Preferably, the composition further comprises a surface tension decreasing agent, for example, ethanol. The xanthan-sorbate

carrier is utilized to maintain the bacteria in a modified state, wherein they are ready to germinate upon contact with a food surface.

[00048] A surfactant is also advantageously utilized with the composition to break the surface tension of water, so that the composition will spread more easily across a wet surface. This allows the composition and the components therein to be more easily spread across and onto the surface, including into existing biofilms where the bacteria of the composition can begin to populate the surface. Preferably the surfactant is a non-ionic amphoteric surfactant. Further suitable surfactants include: C13 alcohol 9 mole ethoxylate, Coconut diethanolamide, C9-11 alcohol 6 mole ethoxylate, and PEG5-cocommonium methyl sulphate. One of ordinary skill in the art will recognize other suitable non-ionic surfactants suitable for achieving the activity described herein.

[00049] An opacifier may be suitably added to the composition, for example styrene/acrylamide copolymer 2-propenamide. Other known opacifiers may be suitably used as known to one of ordinary skill in the art.

[00050] A preferred embodiment of the invention comprises a concentrated aqueous composition comprising *Bacillus subsilis* and *Vaccinium macrocarpon* proanthocyanidins, wherein the biocidal activity is mediated by natural enzymes and antibiotics formed *in situ*. The composition is formulated into a stable concentrate by generating a carrier of water, ethanol, xanthan gum, sorbate, an opacifier, and a fragrance. Spores of *Bacillus subsilis* and optionally of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are fermented or otherwise strained to induce sporulation, wherein the spores can be collected for use. The spores are bound to dendritic salt and dispersed into the carrier as generated above. The carrier and spore concentrate is then measured for pH and modified with HCl to a pH of about 4.5 to 6.0. The acidic pH maintains the spores in a state ready for germination, wherein

the xanthan-sorbate based carrier creates a thicker than water carrier for suspending the spores into the carrier.

[00051] In preferred embodiments, the composition is manufactured as concentrate, such as described above, that can be suitably diluted for application to a surface. A preferred diluent ration is 20:1, with water as the diluent. However, other suitable aqueous solvents may be suitably utilized as a diluent based on the particular needs of the application. Certain alcohol/water based diluents therefore may be advantageous as a mechanism to reduce surface tension in some embodiments. While 20:1 is a preferred dilution ratio, suitable dilutions include a ratio from 100:1 to 0.1:1 and all ratios in between. In certain embodiments, the concentrate can also be utilized in an undiluted form.

[00052] Accordingly, application of a composition as described herein provides for the artificial introduction of benign biofilms and may offer a new paradigm for both hygiene and infection control.

[00053] Application of the diluted composition is achieved by direct application to a surface, such as spray and wipe. For example, the diluted composition can be applied via a spray mist, or poured, or applied to a cloth and applied to a surface for decontamination. Where the surface is relatively small, manual application is appropriate. Furthermore, the manual application and mechanical wiping of the surface with a cloth or material may further advantageously remove surface contaminants through the mechanical action, as well as mechanically disrupt the biofilms present on a contaminated surface. The composition, now being applied, can remove pathogenic bacterial through competitive exclusion, including consumption of water, nutrients and even the pathogenic microbes themselves. Continued mechanical application of the composition will continue to additional mechanical removal of exposed pathogens, but also through the continued deposit of probiotic agents from the composition, thereby inhabiting the open spaces. Therefore, the deposit of the composition to

the surface will begin the process to starve and crowd out pathogenic bacteria and replace such pathogenic biofilm with benign biofilms generated by the bacteria in the composition.

[00054] Furthermore, upon application of the composition to a surface, the bacteria in the composition begin generating ample amounts of the biosurfactant surfactin, as well as biocidal concentrations of subtilisin and lanthionine-containing peptide antibiotics which competitively exclude pathogenic species. Furthermore, in situ production of α -amylase enzymes work to detach biofilm exopolysaccharide components.

[00055] Additional mechanism for application of the composition includes spraying the composition on a surface without mechanical wiping of the surface. For example, in a large contaminated area, application may be completed through a spraying or aeration system, fogger, or other similar mechanism. In certain embodiments, therefore, an appropriate carrier may be further admixed into the invention to aid in application through these means. Industrial scale applications may also be completed through industrial spray systems through ground or aerial vehicles, which can distribute large amounts of diluted materials onto surfaces that require biofilm control.

[00056] Therefore, the composition may be suitably utilized in a method of controlling biofilm on a surface by applying to said surface a composition comprising the *B. subtilis* spores, surfactant, A. proanthcyanidin, in an appropriate carrier. Wherein the method further comprises wiping said surface clean of said composition.

[00057] Therefore, it is further suitable to use the compositions as described herein for the purposes of treating a surface to introduce a benign biofilm. Furthermore, an appropriate use of the compositions is for reducing the occurrence of gram-negative bacteria on a surface. Furthermore, an appropriate use of the composition is for reducing a pathogenic biofilm on a surface by applying said composition to said surface, wherein the bacteria in said

composition will out compete the pathogenic bacteria, degrade the chemical bonds on the pathogenic bacteria biofilm, and through quorum sensing.

[00058] Example 1:

[00059] A composition as described herein was used in a healthcare setting with significant biofouling of washrooms. Swabs were sent for microbiological enumeration before the introduction of the composition to surfaces of several locations. The composition was administered daily for 21 days and swabs were collected again after treatment of these surfaces after 21 days. Before and after results of these tests are described in more detail below and the results shown in Tables 1-3.

[00060] The composition's active ingredients are a blend of *B. subtilis* spores at a concentration of 1×10^8 CFU ml⁻¹, surfactant, and cranberry A-proanthcyanidin in a suitable buffer and carrier. Non-active ingredients included an opacifier, fragrance and buffer. The composition can be advantageously manufactured as a concentrate and diluted for subsequent use of administration to a surface. This concentrated format of the composition was automatically diluted 20:1 (with water) in a Venturi-based chemical proportioner to assure consistency of dilution.

[00061] The composition tested comprises *B. subtilis* strains selected from the group consisting of the following strains: 1A5, 1A28, 1A84, 1A122, 1A698, 1A699, 1A751, and 1A752, and combinations thereof to a concentration of 1×10^8 CFU ml⁻¹. The strains are combined with a surfactant and cranberry A-proanthcyanidin, and further comprising an opacifier, a buffer, and optionally a fragrance. This concentrate can be further diluted 20:1 in an aqueous medium and applied to a surface.

[00062] In preferred embodiments, the *B. subtilis* strains are selected from the group consisting of: 1A5, 1A28, 1A84, 1A122, 1A698, 1A699, 1A751, and 1A752, and combinations thereof according to the BGSC Accession number. Further strains of *B. subtilis*

may be advantageously utilized in certain embodiments. Furthermore, the inclusion of additional strains of *B. subtilis* outside of those enumerated above does not destroy the efficacy of the composition when used at a concentration of less than 50%, less than 25% of the total amount of the *B. subtilis* spores in the composition. Preferably, additional strains are less than 20%, less than 10%, less than 5%, or less than 1% of the total concentration of the *B. subtilis* strains in the composition.

[00063] The selected strains are prodigious producers of surfactin. Furthermore, the same strain or additional strains may be advantageously added for production of biocidal concentrations of subtilisin, lanthionine-containing peptide antibiotics and α -amylase enzymes.

[00064] A concentrate of the composition was generated and diluted 20:1 in water for application to the tested surfaces.

[00065] *1) Microbiological enumeration before introduction of the composition*

[00066] Streak plates from swab samples taken **before** introduction of the composition showed a large number and variety of bacterial colonies both on nutrient agar and MAC with presumptive Gram-negative strains being isolated from 7 of the 9 samples.

[00067] Bacterial counts on these samples showed high bacterial loads, ranging from 10^4 to 10^8 cfu / swab for total counts and from 10^4 to 10^7 cfu / swab for Gram-negative counts.

[00068] *2) Microbiological enumeration after introduction of the composition*

[00069] Conversely, streak plates from samples taken **after** treatment with the composition produced lower numbers of colonies, typically 10^3 to 10^4 cfu /swab.

[00070] Two of the three locations swabbed only showed colonies with morphologies matching those of *Bacillus subtilis*. One other Gram-positive coccus was isolated in swabs from the shower drain in the washroom.

[00071] Gram-negative bacteria were not isolated from any of the sampling sites following treatment, either on the streak plates or those for bacterial counts.

[00072] *3) Presumptive Identification of Bacteria*

[00073] Gram staining, oxidase and catalase testing, and use of selective agars, confirmed the presence of a variety of species in samples taken before treatment, and suggested the presence of clinically relevant species including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus*. Gram-positive rods (assumed to be *Bacillus* on the basis of colony morphology, catalase production and spore stains using a method based on that of Schaeffer and Fulton, modified by Ashby) were also present in some samples.

[00074] After treatment only *Bacillus* was found in two of the three locations. In the third *Bacillus* dominated the samples taken and only other organism isolated was a small quantity of *S. aureus* which was shown not to be MRSA.

[00075] **Advantageous effects of treating surfaces with the composition**

[00076] Anecdotal reports from users suggested areas were visibly cleaner, more pleasant to use, and that the malodours had disappeared after the introduction of the composition. Indeed, this real-world testimony is corroborated by the examples tested below, wherein significant to complete removal of certain bacteria from the test locations were achieved by application of the composition of the present disclosure.

[00077] Microbiological enumeration of the effect of the administration of the composition of the normal flora of the washroom proved the overall bacterial load had decreased and the Gram negative species found in the samples taken before the introduction of the composition had been completely excluded in under 21 days. The Gram-positive *Enterococcus* isolated in the original tests, was also not detected after the introduction of the composition to these surfaces.

[00078] The only other organism detected after treatment was *S. aureus* in a shower tray drain which is unremarkable given 20% of the population are permanently colonised with *S. aureus* and a further 60% are intermittent carriers (Foster, 2004). The strain isolated did not exhibit methicillin resistance.

[00079] The bacterial element of the composition in a preferred embodiment is a prolific producer of antibiotics effective against a range of pathogens including *S. aureus*, *E. coli*, *Helicobacter pylori* (Pinchuk et al., 2001), *Campylobacter* (Sorokulova, Kirik and Pinchuk, 1997) and one lanthionine-containing peptide antibiotic (lantibiotic) produced, subtilin, has a broad spectrum antimicrobial activity – by forming transmembrane pores it depolarises energized bacterial cytoplasmic membranes. Using this activity might offer an alternative to current methods for controlling *C. diff* spores: there is already evidence that using *B. subtilis* as a probiotic dietary supplement is effective against *Clostridium difficile*-associated disease (Peys et al., 2007).

[00080] Accordingly, a preferred embodiment is a composition comprising a blend of *B. subtilis* spores at a concentration of 1×10^8 CFU ml⁻¹, surfactant and cranberry A. proanthocyanidin and further comprising an opacifier, fragrance and buffer which forms a concentrate that can be diluted 20:1 in a Venturi-based chemical proportioner to assure consistency of dilution. The resulting composition, when applied to a surface creates transmembrane pores that depolarises energized bacterial cytoplasmic membranes. Accordingly, as compared to surface treating compositions, such as bleach or alcohols, the composition provides spores that continually manufacture and administer antimicrobial compounds.

[00081] In particularly preferred embodiments, the composition breaks down biofilms containing bacteria and destroys these bacteria wherein they are unable to re-colonize a surface, unlike such products like alcohols or bleach based products.

[00082] Materials

[00083] ¼ strength Ringers solution, nutrient agar (NA) and MacConkey No3 agar (AC) were purchased from LabM Ltd., Bury, UK and prepared according to the manufacturer's instructions.

[00084] Potassium hydroxide and oxidase test strips were purchased from Sigma Aldrich Ltd., Poole, UK, and the Color Gram 2 kit and ID Color Catalase reagent were purchased from Biomérieux Ltd., Basingstoke, UK.

[00085] Cetrimide agar (CET), Mannitol salt agar (MSA), *Pseudomonas* agar (PSA), Slanetz & Bartley Medium (SBM), Tryptone bile X-glucuronide medium (TBX), Xylose lysine desoxycholate agar (XLD) and Brilliance MRSA Agar (BMA) were purchased from Oxoid Ltd., Basingstoke, UK. CET, TBX and XLD were purchased as ready-prepared agar plates, whereas PSA and SBM were prepared according to the manufacturer's instructions.

[00086] Methods

[00087] Concentrated solution of the composition described herein was generated.

[00088] Xanthan gum and sorbate (potassium) are combined into 50% final volume of water and mixed at high speed until dispersed. The mixture is chilled slowly to 4°C and left overnight for stability. The mixture is then re-heated to 18°C with a water jacket or at ambient temperature.

[00089] Surfactant is added to the above mixture to stirred until dissolved. Thereafter, the resultant mixture is again chilled slowly to 4°C and left overnight. The mixture is re-heated to 18°C with a water jacket or at ambient temperature.

[00090] An opacifier is dissolved in a suitable volume of water, and added to the above mixture until homogeneous. The resultant mixture is again chilled slowly to 4°C and left overnight. The mixture is then re-heated to 18°C with a water jacket or at ambient temperature.

[00091] Alcohol (C2-C12 – ethanol preferred) to the mixture and mixed until homogeneous. The resultant mixture is again chilled slowly to 4°C and left overnight. The mixture is then re-heated to 18°C with a water jacket or at ambient temperature.

[00092] Bacterial endospores of *Bactillus subtilis* on dendritic salt carrier are mixed into the mixture and homogenized for 30 minutes, before adding the resultant mixture of water. The resultant mixture is again chilled slowly to 4°C and left overnight. The mixture is then re-heated to 18°C with a water jacket or at ambient temperature.

[00093] A mixture of *Vaccinium macrocarpon* A. proanthocyanidines is further admixed into the mixture at 18°C and homogenized for 30 minutes. The resultant mixture pH is adjusted to 4.5 +/- 0.1 with an appropriate acid, e.g. HCl.

[00094] In further embodiments, the pH may be adjusted to 5.5 +/- 0.1, and the pH can be adjusted with an appropriate weak acid such as citric acid or muriatic acid. The acid may be appropriately chosen and added to form a buffer solution at the appropriate pH.

[00095] **Test samples**

[00096] Swab samples from three sites were taken. Three replicates per location were taken using a standardised 10cm x 10cm grid.

[00097] **Location 1** was a section of floor below the gents' urinal area in a washroom.

[00098] **Location 2** was a corner of a shower tray in the washroom.

[00099] **Location 3** was in the drain of a shower tray in the washroom.

[000100] **Enumeration of Swabs**

[000101] Bacterial numbers were then determined using the Miles & Misra drop count method (Miles, Misra and Irwin, 1938). Briefly, swabs were transferred aseptically into 9ml volumes of sterile ¼ strength Ringer's solution and vortexed for 30 seconds. Samples were then serially diluted 1ml to 9ml, five times, in sterile ¼ strength Ringers solution. 10 µl drops of each dilution were then spotted on to over-dried nutrient agar NA plates and MAC plates

before incubation at 30°C for 18 hours (NA) or 37°C for 24 hours (MAC). Plates were then examined for bacterial growth. Numbers of colonies on dilutions showing between 3 and 30 colonies were then counted. By taking into account (a) the number of colonies counted (b) the dilution and (c) the 10 µl volume plated, the number of bacteria per original swab could then be calculated.

[000102] Presumptive Identification of Bacteria

[000103] 1. Isolation of species present

[000104] The initial sample dilution from the enumeration procedure above was also streaked on to NA and MAC agar plates in the conventional manner in order to culture bacterial species present. Plates were incubated at 30°C (NA) or 37°C (MAC) for 48 hours before being examined for bacterial growth. The number of bacterial species on each plate was estimated using a binocular microscope. Individual colony morphologies from NA plates were then re-streaked on to NA plates and incubated in the same manner in order to obtain pure cultures for further investigation.

[000105] Those cultures were then streaked onto selective agar plates which were incubated according to manufacturers' instructions and then examined for bacterial growth and appropriate colour reactions.

[000106] 1.1 Orientation Tests

[000107] Identification commenced with the following orientation tests on each isolate:

1.1.1. Gram stain in the conventional manner using a Color Gram 2 Kit.

1.1.2. Potassium hydroxide test to confirm Gram status; briefly, a loopful of cultures was emulsified in a drop of 3% potassium hydroxide (Sigma Aldrich, Poole, UK) on a clean glass slide. Visible gelling or stringing of the emulsified culture was indicative of a Gram-negative isolate.

1.1.3. Oxidase test (for Gram-negative rods), using oxidase test strips.

1.1.4. Catalase test (for Gram-positive isolates), using ID Color Catalase reagent.

[000108] Selective agars

[000109] According to the results of the orientation tests in section ii) above, isolates were streaked on to the following selective agars:

1.1.5. MSA (for Gram-positive, catalase-positive cocci) – indicates pathogenic staphylococci and distinguishes *Staphylococcus aureus*.

1.1.6. SBM (for Gram-positive, catalase-negative cocci) – indicates *Enterococcus* and *Streptococcus*.

1.1.7. XLD (for Gram-negative, oxidase-negative rods) – indicates enterobacteria such as *Shigella*, *Salmonella* etc.

1.1.8. TBX (for Gram-negative, oxidase-negative rods) – highly specific for *Escherchia coli*.

1.1.9. PSA (for Gram-negative, oxidase-positive rods) – indicates *Pseudomonas* species.

1.1.10. CET (for Gram-negative, oxidase-positive rods) – indicates *Pseudomonas aeruginosa*.

1.1.11. BMA (for presumptive *Staphylococcus aureus*) – indicates methicillin-resistant *S. aureus* (MRSA)

[000110] Example 2: A further test was performed to evaluate the efficacy of *B. subtilis* compositions in reducing sulphite reducing bacteria (SRBs) using the *B. subtilis* compositions.

[000111] The tests were performed according to BSEN1276:2009, chemical disinfectants & antiseptics – quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requires (Phase 2, Step 1), with consideration being given to NACE TM0194.

[000112] Naturally occurring SRBs were isolated from pond sludge and utilized as the test organism culture. 8 ml of a suspension of the SRBs were prepared in a 1/500th nutrient broth, 1 ml Bovine albumin (3.0g/l) was added with 1 ml of a suspension of *B. subtilis* at 10 E8 to 10 E9/ml. Care was taken so that anaerobic conditions were maintained throughout the test.

[000113] Test samples were stored at 4C for 24 hours, with recoveries of SRBs being performed at 5 minutes, 60 minutes, and 24 hours. A control using an SRB suspension containing no *B. subtilis* was performed to compare. Tests were duplicated on two consecutive days.

[000114] Test 1

Bacillus subtilis inoculation level 0 min = 7.2x10 E8/ml

SRBs recovered/ml

Time	Control	Test	Log Reduction
0 Min	9.1x10 E6		
5 min	8.9x10 E6	4.2x10 E6	.33
60 min	8.5x10 E6	4.7x10 E5	1.26
24 hr	7.6x10 E6	1.1x10 E4	2.84

[000115] Test 2

Bacillus subtilis inoculation levels 0 min = 6.4x10 E8/ml

SRBs recovered/ml

Time	Control	Test	Log Reduction
0 min	8.6x10 E7		
5 min	8.8x10 E7	4.5x10 E7	.29
60 min	7.2x10 E7	2.6x10 E7	.45
24 hr	5.9x10 E7	7.0x10 E5	1.92

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Table 1: Swab Counts

Location	Swab no	Before			After		
		Counts cfu/swab		Strains Isolated	Counts/swab		Strains Isolated
		Total	Gram -ve		Total	Gram -ve	
Location 1 Section of floor below the gents' urinal.	1	2.97 x10 ⁷	ND	4	1.33 x10 ⁴	ND	3
	2	9.54 x10 ⁶	ND	5	2.12 x10 ⁴	ND	5
	3	7.92 x10 ⁴	1.51 x10 ⁷	4	2.03 x10 ⁴	ND	2
Location 2 Corner of a shower tray.	4	4.14 x10 ⁸	2.57 x10 ⁷	4	2.70 x10 ⁴	ND	2
	5	3.60 x10 ⁷	3.89 x10 ⁶	4	2.25 x10 ⁴	ND	1
	6	1.89 x10 ⁷	8.46 x10 ⁴	4	9.72 x10 ³	ND	1
Location 3 Shower tray drain.	7	9.36 x10 ⁷	6.66 x10 ⁵	6	1.89 x10 ⁴	ND	4
	8	1.80 x10 ⁷	4.50 x10 ⁶	6	1.08 x10 ⁴	ND	2
	9	1.33 x10 ⁷	3.13 x10 ⁶	3	1.40 x10 ⁴	ND	4

ND = Not Detected, i.e. < 3.00 x 10³ cfu/g.

"Before" plates contained a variety of isolates.

"After" plates contained predominantly colonies with morphology matching *B. subtilis*.

Table 2: Identification Before

Location	Sample	Isolate	Gram	O X	CAT	CET	MSA	PSA	SBM	TBX	XLD	Presumptive Identification
Location 1 Section of floor below the gents' urinal.	1	A	+ R (M)		+							<i>Bacillus sp</i>
		B	+ R (M)		+							<i>Bacillus sp</i>
		C	+ R (M)		+							<i>Bacillus sp</i>
		D	+ R (M)		+							<i>Bacillus sp</i>
	2	A	+ R (L)		+							<i>Bacillus sp</i>
		B	+ R (M)		+							<i>Bacillus sp</i>
		C	+ R (M)		+							<i>Bacillus sp</i>
		D	+ R (M)		+							<i>Bacillus sp</i>
	3	A	- R (S)	-						+++ BC	+++ SC	<i>Escherichia coli</i>
		B	+ R (M)		+							<i>Bacillus sp</i>
		C	+ R (M)		+							<i>Bacillus sp</i>
		D	+ R (M)		+							<i>Bacillus sp</i>
Location 2 Corner of a shower tray.	4	A	- R (S)	+		+, P		+++				<i>Pseudomonas aeruginosa</i>
		B	+ C		+		+++ PC					Coagulase-negative <i>Staphylococcus sp</i> , e.g. <i>S. epidermidis</i>
		C	- R (S)	+		+		+++				<i>Pseudomonas aeruginosa</i>
		D	+ C/CB		-				+++ RC			<i>Enterococcus sp</i>
	5	A	- R (s)	+		+, P		+++				<i>Pseudomonas aeruginosa</i>
		B	+ C/CB		-				+++ RC			<i>Enterococcus sp</i>
		C	- R (s)	+		-		-				Other non-enteric rod, e.g. <i>Aeromonas</i> , <i>Vibrio sp</i>
		D	- R (S)	+		-		+				<i>Pseudomonas sp</i>
	6	A	- R (S)	+		++		+++				<i>Pseudomonas aeruginosa</i>
		B	+ R (S)		+							<i>Bacillus sp</i>
		C	- R (S)	+		-		-				Other non-enteric rod, e.g. <i>Aeromonas</i> , <i>Vibrio sp</i>
		D	n/g									
Location 3 Shower tray drain.	7	A	- R (S)	-						+++ SC	+++ OC	Other Enteric rod, e.g. <i>Citrobacter</i> , <i>Klebsiella</i> , <i>Serratia</i>
		B	- R (S)	+		+++		+++				<i>Pseudomonas aeruginosa</i>
		C	- R (S)	-						++	-	Inconclusive, likely other enteric rod
		D	+ R (M)		+							<i>Bacillus sp</i>
		E	+ R (S)		+							<i>Bacillus sp</i>
		F	NSG									
	8	A	- R (S)	+		++ P		+++				<i>Pseudomonas aeruginosa</i>
		B	- R (S)	+		++ P		+++				<i>Pseudomonas aeruginosa</i>
		C	- R (S)	-						++	-	Inconclusive, likely other enteric rod
		D	+ R (S)		+							<i>Bacillus sp</i>
		E	+ R (S)		+							<i>Bacillus sp</i>
		F	NSG									
	9	A	- R (S)	+								<i>Aeromonas</i> , <i>Pseudomonas</i> , <i>Vibrio sp</i>
		B	- R (S)	-						-	-	Inconclusive, likely other enteric rod
		C	NSG									

Table 3: Identification After

Location	Sample	Isolate	Gram	O X	CAT	CET	MSA	PSA	SBA	TBX	XLD	BMA	Presumptive Identification
Location 1 Section of floor below the gents' urinal.	1	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
		C	+ R (M)		+								<i>Bacillus sp</i>
	2	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
		C	+ R (M)		+								<i>Bacillus sp</i>
		D	+ R (M)		+								<i>Bacillus sp</i>
		E	+ R (M)		+								<i>Bacillus sp</i>
	3	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
Location 2 Corner of a shower tray.	4	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
	5	A	+ R (M)		+								<i>Bacillus sp</i>
	6	A	+ R (M)		+								<i>Bacillus sp</i>
Location 3 Shower tray drain.	7	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
		C	+ C		+		+++ Y					-	<i>Staphylococcus aureus</i> , not MRSA
		D	+ C		+		+++ Y					-	<i>Staphylococcus aureus</i> , not MRSA
	8	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ C		+		+++ Y					-	<i>Staphylococcus aureus</i> , not MRSA
	9	A	+ R (M)		+								<i>Bacillus sp</i>
		B	+ R (M)		+								<i>Bacillus sp</i>
		C	+ C		+		+++ Y					-	<i>Staphylococcus aureus</i> , not MRSA
		D	+ C		+		+++ Y					-	<i>Staphylococcus aureus</i> , not MRSA

Gram, Gram stain; OX, Oxidase test; CAT, Catalase test

S, Short; M, Medium; L, Long; n/g, no growth

BMA, Brilliance MRSA Agar; CET, Cetrinide Agar; MSA, Mannitol Salt Agar; PSA, Pseudomonas Agar;

SBA; Slanetz & Bartley Medium; TBX, TBX Medium; XLD; XLD Medium

-, negative growth; +, ++, +++, degree of positive growth.

BC, Blue colonies; OC, Opaque colonies; PC, Pink colonies; RC, Reddish colonies;

SC, Straw-coloured colonies

P, evidence of pigment production

Y, Yellowing of agar

NSG, Not sufficiently grown

Claims

1. A biocide composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH.
2. A biocide composition comprising an effective amount of *Bacillus subtilis* and *Vaccinium macrocarpon* proanthocyanidins.
3. The composition of claim 2 further comprising a suitable surfactant.
4. The composition of claim 3 further comprising an opacifier, a buffer, and a fragrance.
5. The composition of claim 3 wherein the suitable surfactant is a non-ionic and amphoteric surfactant.
6. The composition of claim 3 comprising two or more surfactants.
7. The composition of claim 3 further comprising a xanthan-sorbate carrier.
8. The composition of claim 7 further comprising a surface tension decreasing agent.
9. The composition of claim 2 wherein the effective amount of the *Bacillus subtilis* is 1×10^8 cfu/ml.
10. The composition of claim 2 further comprising an effective amount of *Bacillus licheniformis*.
11. The use of the composition of claim 2 to control environmental pathogenic bacteria.
12. The composition of claim 2 further comprising natural antibiotics.
13. The composition of claim 2 further comprising lantibiotics.
14. The use of the composition of claim 2 to digest organic soils.
15. The use of the composition of claim 2 to disrupt biofilm formation.
16. The use of the composition of claim 2 to mediate biocidal activity.

17. A composition suitable for artificially generating a benign biofilm comprising an effective amount of *Bacillus subtilis*, *Vaccinium macrocarpon* proanthocyanidins, a suitable carrier, and a surfactant.

18. The composition of claim 17 wherein the suitable carrier is a xanthan-sorbate carrier.

19. Use of the composition of claim 17 to generate a benign biofilm.

20. A method of disrupting an existing biofilm comprising:

- a. Applying a composition comprising an effective amount of *Bacillus subtilis*, *Vaccinium macrocarpon* proanthocyanidins, a suitable carrier, and a surfactant to surface for a sufficient time to transfer the *Vaccinium macrocarpon* proanthocyanidins to said existing biofilm, wherein said *Vaccinium macrocarpon* proanthocyanidins disrupt said biofilm surface by reducing the mobility of existing bacteria and:
- b. Generating a benign biofilm surface from colonized *Bacillus subtilis* from said application.

21. The method of claim 20 wherein said suitable carrier is a xanthan-sorbate carrier.

22. A biocide composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH.

23. A biofilm control composition comprising one or more strains of *Bacillus subtilis*, which are prodigious producers of natural lantibiotics and other biocidal moieties, acting synergistically with a suite of biologically-active extracts; wherein, in combination with suitable surfactant and A. proanthocyanidin components, said natural lantibiotics and biocidal

moieties provide a means for controlling biofilms in food production and healthcare by – *inter alia* - excluding pathogens as well as direct and indirect biocidal activity.

24. A method for reducing the occurrence of gram-negative bacteria on a surface comprising:

- a. Applying to a surface, a composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH; and
- b. mechanically removing the composition from the surface to remove contaminants removed by the composition.

25. A method of reducing a pathogenic biofilm on a surface comprising:

- a. applying a composition comprising a blend of spores of *Bacillus subtilis* strains and proanthocyanidines from *Vaccinium macrocarpon*, wherein said *Bacillus* spores are bound to a dendritic salt and admixed into a carrier of xanthan gum, potassium sorbate, a C2-C12 alcohol, and a surfactant, and modified to a pH of 4.5 +/- 0.1 with an appropriate acid to form a suitable buffer solution at said pH to a surface;
- b. forming colonies of said bacteria from said composition on said surface so as to out compete the pathogenic bacteria on said surface;
- c. degrading the chemical bonds on the pathogenic bacteria biofilm with the proanthocyanidines; and
- d. reducing the numbers of pathogenic bacteria through quorum sensing.

26. Use of the composition of claim 22 for reducing the number of colony forming units of gram-negative bacteria on a surface.

A. CLASSIFICATION OF SUBJECT MATTER**A01N 63/02(2006.01)I, A01N 25/30(2006.01)I**

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

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
A01N 63/02; A61K 36/185; A61K 31/353; A61K 31/352; A61K 36/45; A01N 25/30Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: biofilm, bacillus subtilis, spore, vaccinium macrocarpon, proanthocyanidin, dendritic salt xanthan gum, potassium sorbate, alcohol, surfactant**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010-078660 A1 (THE ROYAL INSTITUTION FOR THE ADVANCEMENT OF LEARNING/MCGILL UNIVERSITY) 15 July 2010 See paragraphs [0010], [0073] and claims 1-2, 4, 8, 11, 30-31.	1-26
A	ULREY et al., "Cranberry proanthocyanidins have anti-biofilm properties against Pseudomonas aeruginosa" BMC Complementary and Alternative Medicine, Vol.14, Article No.499, Internal pages 1-13 (2014) See the whole document.	1-26
A	RANE et al., "Cranberry-derived proanthocyanidins prevent formation of Candida albicans biofilms in artificial urine through biofilm- and adherence-specific mechanisms" Journal of Antimicrobial Chemotherapy, Vol. 69, No.2, pp. 428-436 (10 October 2013) See the whole document.	1-26
A	KOO et al., "Influence of cranberry proanthocyanidins on formation of biofilms by Streptococcus mutans on saliva-coated apatitic surface and on dental caries development in vivo" Caries Research, Vol.44, pp.116-126 (Epub. 16 March 2010) See the whole document.	1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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INTERNATIONAL SEARCH REPORT

International application No.

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