(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
06 April 2023 (06.04.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/053073 A1

(51) International Patent Classification:
A61K 35/744 (2015.01) *A61K 47/26* (2006.01)
A61P 31/04 (2006.01) *C12R 1/46* (2006.01)

(21) International Application Number:
PCT/IB2022/059321

(22) International Filing Date:
30 September 2022 (30.09.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2021903132 30 September 2021 (30.09.2021) AU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) Title: PROBIOTIC ENHANCERS AND USES THEREOF

(57) Abstract: Described herein is a method of improving the inhibitory profile of Streptococcus salivarius comprising formulating the S. salivarius in a composition comprising an effective amount of a supplemental saccharide. Also described herein are compositions comprising Streptococcus salivarius and supplemental saccharide, methods of inhibiting microorganisms using such compositions, therapeutic formulations comprising such compositions, and methods of treating or preventing diseases using such compositions and therapeutic formulations.

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PROBIOTIC ENHANCERS AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention broadly relates to methods for enhancing the effectiveness of *Streptococcus salivarius* probiotics, and to compositions useful in such methods.

BACKGROUND TO THE INVENTION

[0002] Probiotics are live microorganisms that may provide various health benefits when consumed by, or administered to a subject. For example, *Lactobacillus* spp., and *Bifidobacterium* spp. are well known for use in maintaining or improving gut health.

[0003] A growing probiotics market has led to an increased focus on how to enhance the effectiveness of probiotic strains. To date, much of the focus has been on how prebiotics can alter the gut microbiome, or increase the effectiveness of probiotics useful for gut health. As a result of that work, various prebiotics which induce the growth or activity of gut probiotics have been identified. Such prebiotics include nondigestible fibre compounds, resistant starches, arabinogalactans, and oligosaccharides such as inulin and galacto-oligosaccharides.

[0004] Prebiotics were originally described as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves human health". By this definition, only a limited number of carbohydrates fit the criteria and they were considered for the promotion of bacteria residing in the colon. Since this description was conceived prebiotics have been defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". The currently accepted definition excludes microbiota exclusively present in the oral cavity, however.

[0005] WO2016172658 describes use of a microbiome regulator such as a sugar or sugar alcohol for improving the growth and/or colonisation of beneficial bacteria in the large or small intestine. WO2004074496 describes the use of galacto-oligosaccharides for increasing beneficial bacteria in the gastrointestinal tract. US20200030366 describes methods for treating or preventing colonisation by gastrointestinal pathogenic microorganisms, comprising administering dietary fibre such as inulin. The compositions

may also comprise prebiotics which include a range of sugars/saccharides such as sucrose, maltose, lactose, fructose, galactose, glucose, raffinose, mannose, ribose, and trehalose.

[0006] More recently, probiotics for oral application or orally targeted probiotics have been identified. *S. salivarius* probiotics are known for use against various oral pathogens. For example, *S. salivarius* strain K12 ("K12") is documented for use in preventing or treating ear, nose and throat infections such as those caused by *Streptococcus pyogenes* (see for example WO2001027143 Blis Technologies Ltd). K12 is also known for use in the treatment of halitosis caused by anaerobic bacteria (see for example WO2005007178 Blis Technologies Ltd). *S. salivarius* strain Mia (herein "M18") is known for use in the treatment of dental caries caused by *Streptococcus mutans* (see for example WO2003070919 Blis Technologies Ltd).

[0007] US20190336428 describes selectively increasing the growth of beneficial bacteria in the oral cavity using a saccharide selected from D-turanose, D-melezitose, D-lactitol, myo-inositol, and N-acetyl-D-mannosamine. WO2012065811 describes nutritional compositions for children that may comprise probiotics and prebiotics. US20160166501 describes oral compositions comprising *Lactobacillus helveticus* for use in oral hygiene. The compositions may also comprise other probiotic strain and excipients. WO2007144334 describes a composition for treating otitis media comprising a probiotic *Lactobacillus* strain and a bacterial strain capable of exerting bacteriostatic effects such as *S. salivarius* K12. The composition may be in the form of an infant formula comprising lactose.

[0008] US20190343899 describes a hard candy or toffee composition comprising prebiotics, prepared at high temperatures. WO2017129639 describes an infant formula comprising oligosaccharides.

[0009] Simple sugars are known to be used as prebiotics to promote the growth of bacteria by being consumed as an energy source. As bacteria utilise these sugars, they may produce organic acids as a by-product. These acids can have a weak non-selective inhibitory effect on the growth of other bacteria. Further, if used in the oral cavity, the production of acidic by-products can lower the environmental pH and cause the erosion of tooth enamel which can progress to tooth decay. A more acidic environment also promotes the growth of harmful dental pathogens such as *S. mutans*.

[0010] There is a need to identify alternatives to common sugars to promote antimicrobial activity in probiotics without significant influence on pH. There is also a

need to identify alternatives to common sugars which enhance colonisation efficacy of probiotics to impart health benefits. There is also a need for compositions that can enhance the activity of probiotics in areas other than the gastrointestinal tract. It is an object of the present invention to go some way to meeting any one or more of these needs; and/or to at least provide the public with a useful choice.

[0011] Other objects of the invention may become apparent from the following description which is given by way of example only.

[0012] Any discussion of documents, acts, materials, devices, articles, or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date.

SUMMARY OF THE INVENTION

[0013] In a first aspect, the invention provides a method of improving the inhibitory profile of *Streptococcus salivarius* comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

[0014] In a second aspect, the invention provides a method for upregulating one or more genes in *Streptococcus salivarius*, comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

[0015] In a third aspect, the invention provides a method of inhibiting a skin, dental, oral, mucosal and/or ENT microorganism, the method comprising contacting the microorganism with a composition comprising *Streptococcus salivarius* and an effective amount of a saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[0016] In a fourth aspect, the invention provides a method for increasing production of one or more of a lantiobiotic peptide, bacteriocin or urease by *Streptococcus salivarius*, comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof, and the production is increased relative to a composition lacking the supplemental saccharide.

[0017] In a fifth aspect, the invention provides a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide for use in improving the inhibitory profile of the *Streptococcus salivarius*,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[0018] In a sixth aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, and raffinose in an amount of 2 to 3% by weight.

[0019] In a seventh aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, and galactose in an amount of 0.25 to 0.75% by weight.

[0020] In an eighth aspect, the invention provides a composition comprising *Streptococcus salivarius* M18, and raffinose in an amount of 2 to 3% by weight.

[0021] In a ninth aspect, the invention provides a composition comprising *Streptococcus salivarius* M18, and galactose in an amount of 0.25 to 0.75% by weight.

[0022] In a tenth aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, and raffinose in an amount of 2 to 3% by weight.

[0023] In an eleventh aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, and galactose in an amount of 0.25 to 0.75% by weight.

[0024] In a twelfth aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, raffinose in an amount of 2 to 3% by weight, and galactose in an amount of 0.25 to 0.75% by weight.

[0025] In a thirteenth aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

[0026] In a fourteenth aspect, the invention provides a composition comprising Streptococcus *salivarius* M18, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

[0027] In a fifteenth aspect, the invention provides a composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

[0028] In a sixteenth aspect, the invention provides a therapeutic formulation comprising the composition of any one of the fifth to fifteenth aspects.

[0029] In a seventeenth aspect, the invention provides a method of treating or preventing a disease or disorder comprising administering to subject in need thereof a composition of any one of the fifth to fifteenth aspects, or a therapeutic formulation of the sixteenth aspect.

[0030] In an eighteenth aspect, the invention provides a method of inhibiting a microorganism sensitive to Blis-producing *S. salivarius*, the method comprising administering to subject in need thereof a composition of any one of the fo fifth to fifteenth aspects, or a therapeutic formulation of the sixteenth aspect.

[0031] In a nineteenth aspect, the invention relates to use of *Streptococcus salivarius* and a supplemental saccharide in the manufacture of a medicament for:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius,*

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[0032] In a twentieth aspect, the invention provides a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide for use in:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius,*

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[0033] In a twenty-first aspect, the invention provides a method of manufacturing a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide, the method comprising:

(a) combining *Streptococcus salivarius* with supplemental saccharide, and

(b) mixing to produce a homogeneous blend:

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[0034] In a twenty-second aspect, the invention relates to use of a composition manufactured by the method of the twenty-first aspect for the treatment or prevention of a disease or disorder, or for the inhibition of a microorganism sensitive to Blis-producing *S. salivarius.*

[0035] The following embodiments and preferences may relate alone or in any combination of any two or more to any of the above aspects.

[0036] In various embodiments, the upregulated gene(s) encodes for a lantibiotic peptide or bacteriocin.

[0037] In various embodiments, the upregulated gene(s) encodes for a Class I lantibiotic peptide or Class II bacteriocin.

[0038] In various embodiments, the lantibiotic peptide is *salA, salB, sal9* or a combination thereof.

[0039] In various embodiments, the lantibiotic peptide is *salA, salB*, or a combination thereof.

[0040] In various embodiments, the bacteriocin is *salQ*.

[0041] In various embodiments, the upregulated gene(s) encodes for a subunit of a urease protein.

[0042] In various embodiments, the urease is *ureC*.

[0043] In various embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 70% sequence identity to any one of SEQ ID NOs 15-22, or at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 70% sequence identity to any one of SEQ ID NOs 23-30.

[0044] In various embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 70% identity to any one of SEQ ID NOs 15-22, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs 15-22.

[0045] In various embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 70% identity to any one of SEQ ID NOs 23-30, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs 23-30.

[0046] In various embodiments:

a. at least one of the upregulated gene(s) is a *sal*A gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 15 or 19, or encoding a polypeptide with at least 70% identity to SEQ ID NO 23 or 27;

b. at least one of the upregulated gene(s) is a *sal*B gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 16, or encoding a polypeptide with at least 70% identity to SEQ ID NO 24;

c. at least one of the upregulated gene(s) is a *sal*Q gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 17 or 21, or encoding a polypeptide with at least 70% identity to SEQ ID NO 25 or 29;

d. at least one of the upregulated gene(s) is a *sal*9 gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 20, or encoding a polypeptide with at least 70% identity to SEQ ID NO 28; and/or

e. at least one of the upregulated gene(s) is a *ure*C gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 18 or 22, or encoding a polypeptide with at least 70% identity to SEQ ID NO 26 or 30.

[0047] In various embodiments, the lantibiotic peptide or bacteriocin is a Class I or Class II lantibiotic peptide or bacteriocin.

[0048] In various embodiments, the lantibiotic peptide is *salA, salB, sal9* or a combination thereof.

[0049] In various embodiments, the lantibiotic peptide is *salA, sal*B, or a combination thereof.

[0050] In various embodiments, wherein the bacteriocin is *salQ.*

[0051] In various embodiments, the method increases production of a polypeptide with at least 70% sequence identity to any one of SEQ ID NOs 23-30.

[0052] In various embodiments, at the polypeptide has at least 75% identity to any one of SEQ ID NOs 23-30, preferably at least 80%, 85%, 90%, 95%, or 99% identity to any one of SEQ ID NOs 23-30.

[0053] In various embodiments, the method increases the inhibitory profile of *S. salivarius* against skin, dental, oral, mucosal and/or ENTR microorganisms.

[0054] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *Staphylococcus aureus* spp., *Staphylococcus intermedius spp.*, *Staphylococcus saprophyticus* spp., *Moraxella catarrhalis* spp., *Haemophilus influenzae* spp., *Streptococcus pyogenes* spp., *Pseudomonas aeruginosa* spp., *Streptococcus mutans* spp., *Streptococcus pneumoniae* spp., *Cutibacterium acnes*, *Candida albicans* spp. *Streptococcus sobrin*us spp., *Corynebacterium* spp., *Fusobacterium nucleatum* spp., *Aggregatibacter actinomycetemcomitans* spp., *Porphyromonas gingivalis* spp., *Tannerella forsythia* spp., *Treponema denticola* spp., *P. intermedia* spp., *Prevotella* spp., *Actinomyces viscosus* spp., *Streptococcus equismillis* spp., *Streptococcus dygalactiae* spp., *Streptococcus sanguis* spp., *Staphylococcus cohnii* spp., *B. intermedius* spp., *Atopobium parvulum* spp., *Eubacterium saburreum* spp., *Eubacterium sulci* spp., *Parvimonas micra* spp., *Solobacterium moorei* spp., *Streptococcus agalactiae* spp., *C. minutissimus* spp., *Propionibacterium propionicus* spp., *Streptococcus agalactiae* spp., *Streptococcus dysgalactiae* spp., *Staphylococcus simulans* spp., *Staphylococcus xylosus* spp., Tinea pedis infection causing fungi, *S. salivarius* spp. other than K12 or M18, *Lactococcus lactis* spp., *Staphylococcus epidermidis* spp., *Streptococcus constellatus* spp., *Klebsiella pneumoniae* spp., *Acinetobacter baumanii* spp. or any combination of any two or more thereof.

[0055] In various embodiments, the microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. saprophyticus* ATCC 15305, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *H. influenzae* TW5, *S. pyogenes* M76, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *P. aeruginosa* I2, *S. mutans* OMZ175, *S. pneumoniae* D39, *L. lactis* T-21, *S. epidermidis* 11, *S. constellatus* T-29, *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, or any combination of any two or more thereof.

[0056] In various embodiments, the composition comprises at least about 0.1% by weight of *S. salivarius*.

[0057] In various embodiments, the composition comprises from about 0.1 to about 20% by weight of *S. salivarius*.

[0058] In various embodiments, the composition comprises at least about 1×10^3 cfu/g of *S. salivarius*.

[0059] In various embodiments, the composition comprises from about 1×10^3 to about 1×10^{13} cfu/g of *S. salivarius*.

[0060] In various embodiments, the composition comprises less than about 20% by weight supplemental saccharide.

[0061] In various embodiments, the composition comprises from about 0.1 to about 20% by weight supplemental saccharide.

[0062] In various embodiments, the composition is formulated for oral, dental, nasal, mucosal, topical, or pulmonary administration.

[0063] In various embodiments, the composition is formulated in a slow-release composition.

[0064] In various embodiments, the composition is formulated into a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel spray, deodorant, serum, lotion, balm, moisturiser, pessary, or suppository.

[0065] In various embodiments, the microorganism is a *Streptococcus* or *Staphylococcus* bacteria selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. pyogenes* spp., *S. pneumoniae* spp.; and the *S. salivarius* strain is K12.

[0066] In various embodiments, the *Streptococcus* or *Staphylococcus* bacteria is selected from *S. aureus* A222, *S. saprophyticus* ATCC 15305, *S. mutans* OMZ175, *S. constellatus* T-29, *S. pyogenes* 71-698, and *S. pneumoniae* D39; and the *S. salivarius* strain is K12.

[0067] In various embodiments, the supplemental saccharide is raffinose and is present in the composition in an amount of 0.5 to 15%, or 1 to 12%, or 1.5 to 10%, or 2 to 7%, or 2.5 to 5% by weight.

[0068] In various embodiments, the supplemental saccharide is galactose and is present in the composition in an amount of 0.5 to 15%, or 1 to 12%, or 1.5 to 10%, or 2 to 7%, or 2.5 to 5% by weight.

[0069] In various embodiments, the bacteria are selected from *S. pyogenes* spp., and *S. pneumoniae* spp.; and the *S. salivarius* strain is M18.

[0070] In various embodiments, the bacteria are selected from *S. pyogenes* 71-698, and *S. pneumoniae* D39; and the *S. salivarius* strain is M18.

[0071] In various embodiments, the bacteria are selected from *S. constellatus*, *S. mutans, and S. saprophyticus*; and the *S. salivarius* strain is M18.

[0072] In various embodiments, the bacteria are selected from *S. constellatus* T29, *S. mutans* OMZ175, *and S. saprophyticus* ATCC 15305; and the *S. salivarius* strain is M18.

[0073] In various embodiments, the supplemental saccharide is raffinose and is present in the composition in an amount of 0.25 to 10%, or 0.5 to 8%, or 0.75 to 7%, or 1 to 6%, or 1.25 to 5% by weight.

[0074] In various embodiments, the supplemental saccharide is galactose and is present in the composition in an amount of 0.25 to 10%, or 0.5 to 8%, or 0.75 to 7%, or 1 to 6%, or 1.25 to 5% by weight.

[0075] In various embodiments, the composition comprises one or more of: galactose in an amount of 0.1 to 1%, or 0.2 to 0.8, or 0.25 to 0.75, or at 0.5% by weight, and raffinose in an amount of 0.5 to 5%, or 1 to 4, or 2 to 3, or 2.5% by weight.

[0076] In various embodiments, the composition comprises one or more of: galactose in an amount of about 1.7% by weight; and raffinose in an amount of about 1.25% by weight.

[0077] In various embodiments, the composition comprises one or more of: galactose in an amount of about 0.5% by weight; and raffinose in an amount of about 2.5% by weight.

[0078] In various embodiments, the composition comprises raffinose in an amount of 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3.0 % by weight.

[0079] In various embodiments, the composition comprises galactose in an amount of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 % by weight.

[0080] In various embodiments, the composition comprises raffinose in an amount of 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, or 2.2 % by weight.

[0081] In various embodiments, the composition comprises galactose in an amount of 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.25, 1.3, 1.4, 1.5, 1.6 or 1.7 % by weight.

[0082] In various embodiments, the composition further comprises one or more of a carrier; a tableting aid, including a binder or a lubricant; and a flavouring agent.

[0083] In various embodiments, the therapeutic formulation is formulated for oral, dental, nasal, mucosal, topical, or pulmonary administration.

[0084] In various embodiments, the therapeutic formulation is a slow-release composition.

[0085] In various embodiments, the therapeutic formulation is a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel, spray, deodorant, serum, lotion, balm, moisturiser, pessary, or suppository.

[0086] In various embodiments, the therapeutic formulation is a powder.

[0087] In various embodiments, the therapeutic formulation is a lozenge.

[0088] In various embodiments, the disease or disorder is caused by an oral, dental, mucosal, skin, or ENTR pathogen.

[0089] In various embodiments, the disease or disorder is caused by a pathogenic *Streptococcus* or *Staphylococcus* bacteria.

[0090] In various embodiments, the pathogenic *Streptococcus* or *Staphylococcus* bacteria is selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. pyogenes* spp., and *S. pneumonia*e spp.

[0091] In various embodiments, the disease or disorder is selected from otitis media, sore throat, tooth decay, acute pharyngitis, tonsillitis, pneumonia, chronic obstructive pulmonary disease (COPD), periodontal disease, gingivitis, halitosis, dental caries, sepsis,

meningitis, candidiasis (oral thrush), vaginitis, body odour, acne, actinomycosis, psoriasis, erythrasma, cellulitis, impetigo, atopic dermatitis, bacteraemia, athlete's foot, soft tissue infections, erythema, nosocomial, erythema, SARS-CoV, influenza A, influenza B, and RSV or any combination of any two or more thereof.

[0092] In various embodiments, the microorganism sensitive to Blis-producing *S. salivarius* is selected from *S. salivarius* spp., *S. epidermidis* spp., *S. constellatus* spp., and *L. lactis* spp.

[0093] In various embodiments, the microorganism sensitive to Blis-producing *S. salivarius* is selected from *S. pyogenes* spp., *F. nucleatum* spp., and *P. gingivalis* spp.

[0094] In various embodiments, the subject is a human. In some embodiments, the human is a child.

[0095] In various embodiments, the composition is a cosmetic.

[0096] In various embodiments, the composition is a dietary supplement.

[0097] In various embodiments, the composition is a natural health product.

[0098] In various embodiments, the composition is a complementary medicine.

[0099] In various embodiments, the composition is a lozenge, and the method for manufacturing the composition further comprises a step of pressing the homogeneous blend to produce the lozenge.

[00100] In various embodiments, the inhibitory profile of the *S. salivarius* in the composition is improved relative to a composition lacking the supplemental saccharide.

[00101] In various embodiments, the composition is for use in:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius*.

[00102] The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art

to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

[00103] It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9, and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5, and 3.1 to 4.7) and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

[00104] In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

[00105] To those skilled in the art to which the invention relates, many changes in construction and widely differing embodiments and applications of the invention will suggest themselves without departing from the scope of the invention as defined in the appended claims. The disclosures and the descriptions herein are purely illustrative and are not intended to be in any sense limiting.

[00106] Although the present invention is broadly as defined above, those persons skilled in the art will appreciate that the invention is not limited thereto and that the invention also includes embodiments of which the following description gives examples.

BRIEF DESCRIPTION OF THE FIGURES

[00107] The present invention will be described with reference to the accompanying figures, in which:

[00108] Figure 1 shows the change in the size of ENTR pathogen ZOI size (mm) following a deferred antagonism assay conducted with K12 as the producer organism cultured in the presence of various concentrations of galactose. The control condition was

set to (normalised) 0 mm, any deviation from 0 represents the change in the ZOI size (mm).

[00109] Figure 2 shows the change in the size of ENTR pathogen ZOI size (mm) following a deferred antagonism assay conducted with K12 as the producer organism cultured in the presence of various concentrations of raffinose. The control condition was set to 0mm, any deviation from 0 represents the change in the ZOI size (mm).

[00110] Figure 3 shows the change in the size of ENTR pathogen ZOI size (mm) following a deferred antagonism assay conducted with M18 as the producer organism cultured in the presence of various concentrations of galactose. The control condition was set to 0mm, any deviation from 0 represents the change in the ZOI size (mm).

[00111] Figure 4 shows the change in the size of ENTR pathogen ZOI size (mm) following a deferred antagonism assay conducted with M18 as the producer organism cultured in the presence of various concentrations of raffinose. The control condition was set to 0mm, any deviation from 0 represents the change in the ZOI size (mm).

[00112] Figure 5 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equimolar) against skin pathogen *S. aureus* A222. Raffinose vs (a) Trimix (mixture of equimolar concentrations of the three saccharides galactose, glucose and fructose present in raffinose); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equimolar) against skin pathogen *S. aureus* A222. Raffinose vs individual saccharides.

[00113] Figure 6 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equimolar) against dental pathogen *S. mutans* OMZ175. Raffinose vs (a) Trimix (mixture of equimolar concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equimolar) against dental pathogen *S. mutans* OMZ175. Raffinose vs individual saccharides.

[00114] Figure 7 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equimolar) against Lower respiratory tract pathogen *S. pneumoniae* D39. Raffinose vs (a) Trimix (mixture of equimolar concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equimolar) against Lower respiratory tract pathogen *S. pneumoniae* D39. Raffinose vs individual saccharides.

[00115] Figure 8 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharide (equal percentage weight) against Skin pathogen *S aureus* A222. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against skin pathogen *S. aureus* A222. Raffinose vs individual saccharides.

[00116] Figure 9 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against Dental pathogen *S. mutans* OMZ175. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against Dental pathogen *S. mutans* OMZ175. Raffinose vs individual saccharides.

[00117] Figure 10 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogen *S. pyogenes* 71-968. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against ENTR pathogen *S. pyogenes* 71-968. Raffinose vs individual saccharides.

[00118] Figure 11 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29. Raffinose vs individual saccharides.

[00119] Figure 12 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against lower respiratory tract pathogen *S. pneumoniae* D39. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against lower respiratory tract pathogen *S. pneumoniae* D39. Raffinose vs individual saccharides.

[00120] Figure 13 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogens *S. pyogenes* 71-698, dental pathogen *S. mutans* OMZ175, *S. pneumoniae* D39, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides. Raffinose vs (a) Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides; (c) comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal weight percentage) against range of pathogens and microorganism sensitive to Blis-producing *S. salivarius*. Raffinose vs individual saccharides.

[00121] Figure 14 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against microorganism sensitive to Blis-producing *S. salivarius; S. salivarius* #6, *S. salivarius* 193, *S. salivarius* 20P3. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides); (b) individual saccharides.

[00122] Figure 15 shows the comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against microorganism sensitive to Blis-producing *S. salivarius; S. salivarius* #6, *S. salivarius* 193, *S. salivarius* 20P3. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00123] Figure 16 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several oral and ENTR pathogens *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66 and *S. pyogenes* M74. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00124] Figure 17 shows the comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several oral and ENTR pathogens *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66 and *S. pyogenes* M74. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00125] Figure 18 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several lower respiratory tract pathogens *S. dysgalactiae* Bris 2, *S. dysgalactiae* T277, *S. pneumoniae* D39, *S. pneumoniae* RX1, *S. pneumoniae* PK8. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00126] Figure 19 shows the comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several ENTR / lower respiratory tract pathogens *S. dysgalactiae* Bris 2, *S. dysgalactiae* T277, *S. pneumoniae* D39, *S. pneumoniae* RX1, *S. pneumoniae* PK8. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00127] Figure 20 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several dental pathogens *S. mutans* OMZ175, *S. mutans* ATCC 10449, *S. mutans* D10, *S. mutans* UA159, *S. mutans* FW75, *A. viscosus* T14, *S. sanguis* K11, *S. sobrinus* OMZ176. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00128] Figure 21 shows the comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several dental pathogens *S. mutans* OMZ175, *S. mutans* ATCC 10449, *S. mutans* D10, *S. mutans* UA159, *S. mutans* FW75, *A. viscosus* T14, *S. sanguis* K11, *S. sobrinus* OMZ176. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00129] Figure 22 shows the comparison of stimulation on K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several skin pathogens *S. cohnii, S. simulans, S. aureus* A222, *S. aureus* 20, *S. aureus* 19, *S. aureus* 14. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00130] Figure 23 shows the comparison of stimulation on M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against several skin pathogens *S. cohnii, S. simulans, S. aureus* A222, *S. aureus* 20, *S. aureus* 19, *S. aureus* 14. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00131] Figure 24 shows the comparison of stimulation on dairy free K12 inhibitory effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogens *S. pyogenes* 71-*S. pneumoniae* D39698, dental pathogen *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00132] Figure 25 shows the comparison of stimulation on dairy free M18 inhibitory effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, dental pathogen *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00133] Figure 26 shows the comparison of stimulation of growth of K12 in M17 broth. Raffinose, galactose, Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00134] Figure 27 shows the comparison of stimulation of growth of K12 in M17 broth and inhibitory effect using effect using raffinose vs saccharides (equal percentage weight) against oral and ENTR pathogens *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66 and *S. pyogenes* M74. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00135] Figure 28 shows the comparison of stimulation of growth of K12 in M17 broth and inhibitory effect using effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogens *S. pyogenes* 71-698, *S. pyogenes* M74, *S. pyogenes* M66, *S. pneumoniae* D39, dental pathogen *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T-29. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides glucose, fructose and galactose.

[00136] Figure 28A shows the comparison of stimulation of growth of M18 in M17 broth. Raffinose, Galactose, Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00137] Figure 28B shows the comparison of stimulation of growth of M18 in M17 broth and inhibitory effect using effect using raffinose vs saccharides (equal percentage weight) against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, dental pathogen *S. mutans* OMZ175, skin pathogen *S. saprophyticus* ATCC 15305 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T-29. Raffinose vs Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides glucose, fructose and galactose.

[00138] Figure 29 shows magnified photos of K12 and M18 mucoid morphology when grown in CABCa agar supplemented with raffinose compared to CABCa control.

[00139] Figure 30 shows photos of K12 and M18 (dairy or dairy free) change to mucoid morphology when grown in CABCa agar supplemented with raffinose compared to CABCa control.

[00140] Figure 31 shows photos of K12 and M18 producer streaks and glass slides showing bacterial growth. Dotted black ovals visually shows that a higher amount of mucous is produced in CABCa agar supplemented with raffinose compared to CABCa supplemented with Trimix (mixture of equal weight percentage concentrations of the three saccharides) and CABCa control.

[00141] Figure 32 shows the effect of 2.5% w/w raffinose on the antimicrobial activity of different *S. salivarius* strains against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, dental pathogens *S. sobrinus* OMZ176, *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29..

[00142] Figure 33 shows the effect of 0.5%w/w galactose on the antimicrobial activity of different *S. salivarius* strains against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, dental pathogens *S. sobrinus* OMZ176, *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29.

[00143] Figure 34 shows the effect of raffinose, galactose and their combination on the antimicrobial activity of *S. salivarius* K12 against different gram-negative strains *F. nucleatum* ATCC 25586, *F. nucleatum* FH2, *F. nucleatum* FH3, *P. gingivalis* ATCC 33277, *P. gingivalis* W50, *P. intermedia* ATCC 23611, pathogens implicated in Halitosis.

[00144] Figure 35 shows the effect of raffinose, galactose and their combination on the antimicrobial activity of *S. salivarius* M18 against different gram-negative strains *F. nucleatum* ATCC 25586, *F. nucleatum* FH2, *F. nucleatum* FH3, *P. gingivalis* ATCC 33277, *P. gingivalis* W50, *P. intermedia* ATCC 23611, pathogens implicated in Halitosis.

[00145] Figure 36 shows effect of raffinose, galactose and their combination on the antimicrobial activity of *S. salivarius* K12 freeze dried raw ingredient against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, dental pathogens *S. sobrinus OMZ176*, *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29.

[00146] Figure 37 shows the effect of raffinose, galactose and their combination on the antimicrobial activity of *S. salivarius* M18 freeze dried raw ingredient against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, *M. catarrhalis* TW1, dental pathogens *S. sobrinus* OMZ176, *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganism sensitive to Blis-producing *S. salivarius; S. constellatus* T29.

[00147] Figure 38 shows the effect of raffinose, galactose and their combination on the antimicrobial activity of a combination of *S. salivarius* K12 and *S. salivarius* M18 freeze dried raw ingredient against ENTR pathogens *S. pyogenes* 71-698, *S. pneumoniae* D39, S. *agalactiae* ATCC 12386, *M. catarrhalis* TW1, dental pathogens *S. sobrinus* OMZ176, *S. mutans* OMZ175, skin pathogens *S. saprophyticus* ATCC 15305 and *S. aureus* A222 and microorganisms sensitive to Blis-producing *S. salivarius; S. constellatus* T29 and L. lactis ATCC19435.

Figure 39 shows the effect of supplemental saccharide on the inhibitory activity of *S. salivarius* K12 against pathogens *S. pyogenes* 71-698, *S. pyogenes* 71-679, *S. pyogenes* H13, *S. pyogenes K26, S. pyogenes H29, S. pyogenes* WS02, *S. mutans* OMZ175, *S. mutans* FW75, *S. sobrinus* ATCC 27351, *S. sanguis* K11, *S. agalactiae* ATCC 12386, *A. viscosus* T14, *A. viscosus* ATCC 15987, S. constellatus T29, *S. pyogenes* K7, *S. pneumoniae* D39, *S. pneumoniae* PK8, *S. sobrinus* OMZ176, *S. dysgalactiae* T-148, *S. dysgalactiae* Bris 2, *S. dysgalactiae* T277, *C. auris* ATCC 51966, *S. aureus* 19, in a commercial powder formulation (Split plate method).

[00148] Figure 40 shows a comparison of antimicrobial activity of five compositions: *S. salivarius* K12-containing commercial powder dosage form (Daily Defense Junior)

combined with 0.5% w/w galactose; Daily Defense Junior combined with 2.5% w/w raffinose; Daily Defence Junior combined with 0.5% w/w galactose and 2.5% w/w raffinose; a commercial infant formula combined with *S. salivarius* K12; whole milk powder combined with *S. salivarius* K12.

[00149] Figure 41 shows the relative expression of *salA* in *S. salivarius* K12 with the following saccharides added to the solid medium (CABCa agar); 0.5% w/w galactose; 2.5% w/w raffinose; 0.5% w/w galactose in combination with 2.5% w/w raffinose; 0.5% w/w sucrose; 2.5% w/w sucrose. Data points are means (n = 3± SD). Data points are means (n = 3± SD).

[00150] Figure 42 shows the relative expression of *salB* in *S. salivarius* K12 when different combinations of sugars are added to the solid medium (CABCa agar); 0.5% w/w Galactose; 2.5% w/w raffinose; 0.5% w/w galactose in combination with 2.5% w/w raffinose; 0.5% w/w sucrose; 2.5% w/w sucrose. Data points are means (n = 3± SD).

[00151] Figure 43 shows the relative expression of *salQ* in *S. salivarius* K12 when different combinations of supplemental saccharides are added to the solid medium (CABCa agar); 0.5% w/w Galactose; 2.5% w/w raffinose; 0.5% w/w galactose in combination with 2.5% w/w raffinose; 0.5% w/w sucrose; 2.5% w/w sucrose. Data points are means (n = 3± SD).

[00152] Figure 44 shows the relative expression of Urease (*ureC*) in *S. salivarius* K12 when different combinations of supplemental saccharides are added to the solid medium (CABCa agar); 0.5% w/w galactose; 2.5% w/w raffinose; 0.5% w/w Galactose in combination with 2.5% w/w raffinose; 0.5% w/w sucrose; 2.5% w/w sucrose. Data points are means (n = 3± SD).

[00153] Figure 45 shows the average percentage of *S. salivarius* K12 (of total *S. salivarius*) in saliva samples obtained from the groups of participants using lozenges comprising *S. salivarius* K12 without supplemental saccharides (G1), with galactose (G2), raffinose (G3) and raffinose and galactose (G4) at 1 hours, 8 hours and 24 hours after dissolving first lozenge in the mouth, and at 48 hours after a 7-day period of daily dissolving a lozenge in the mouth.

DETAILED DESCRIPTION OF THE INVENTION

[00154] The role of saccharides, and in particular digestible saccharides such as mono-, di- and tri-saccharides for stimulating growth and/or activity of oral bacteria has not been fully explored. The inventors sought to better understand the role of such saccharides on oral probiotic performance.

[00155] The metabolism of certain simple sugars, such as glucose, can act to supress the activation/transcription of certain genes including bacteriocin genes in some bacteria. This effect is commonly known as catabolite repression (see: Görke & Stülke, *Nature Reviews Microbiology* (2008) 6, 613-624). While assessing whether other sugars had a similar repressive effect, the inventors surprisingly found that the supplementation of the growth medium with certain other saccharides (such as lactose) had the opposite effect, enhancing the inhibitory spectrum of some strains of bacterial species, including but not limited to *Streptococcus salivarius*.

Definitions

[00156] The following definitions are presented to better define the present invention and as a guide for those of ordinary skill in the art in the practice of the present invention. Unless otherwise specified, all technical and scientific terms used herein are to be understood as having the same meanings as is understood by one of ordinary skill in the relevant art to which this disclosure pertains.

[00157] The general chemical and biological terms used, for example, in the formulae herein have their usual meanings.

[00158] Examples of definitions of common terms in microbiology, molecular biology and biochemistry can be found in Methods for General and Molecular Microbiology, 3rd Edition, C. A. Reddy, et al. (eds.), ASM Press, (2008); Encyclopaedia of Microbiology, 2nd ed., Joshua Lederburg, (ed.). Academic Press, (2000); Microbiology By Cliffs Notes, I. Edward Alcamo, Wiley, (1996); Dictionary of Microbiology and Molecular Biology, Singleton et al. (2d ed.) (1994); Biology of Microorganisms 11th ed. Brock et al., Pearson Prentice Hall, (2006); Biodiversity of Fungi: Inventory and Monitoring Methods, Mueller et al. Academic Press, (2004); Genes IX, Benjamin Lewin, Jones & Bartlett Publishing, (2007); The Encyclopaedia of Molecular Biology, Kendrew et al. (eds.), Blackwell Science Ltd., (1994); and Molecular Biology and Biotechnology: a Comprehensive Desk Reference, Robert A. Meyers (ed.), VCH Publishers, Inc., (1995).

These sources also provide standard microbiological, molecular biology and biochemistry protocols and procedures that can be used to perform the present invention.

[00159] The term "comprising" as used in this specification and claims means "consisting at least in part of". When interpreting each statement in this specification and claims that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise", "comprised" and "comprises" are to be interpreted in the same manner.

[00160] As used herein the term "and/or" means "and" or "or", or both.

[00161] As used herein "(s)" following a noun means the plural and/or singular forms of the noun.

[00162] The term "subject" as used herein refers to an animal, including humans, and non-human animals such as dogs, pigs, cats, horses, sheep, cows, chickens, fish, and other domestic and farm animals.

[00163] The term "treatment" as used herein refers to a subject undergoing prophylactic or therapeutic treatment. Prophylactic treatment includes treatment to prevent or reduce the likelihood or severity of infection, or to inhibit or control a microbial population. Treatment can be to inhibit or reduce a microbial population in a subject. Treatment can also be provided to an infected subject to decrease the severity of, or to reduce or eliminate an infection or its associated symptoms. The subject to be treated may be at any age, e.g., infant, childhood, adolescence, adulthood, or elderly. Where a "patient," "individual," or "host" is referred to it is synonymous with the term "subject".

[00164] As used herein, a "supplemental saccharide" is a saccharide that improves the inhibitory profile and/or mucoid properties of a bacterial species, such as *S. salivarius*.

[00165] As used herein "improving the inhibitory profile of *Streptococcus salivarius*" means enhancing the potency of inhibition, and/or increasing the spectrum of inhibitory activity of the *S. salivarius* compared to the *S. salivarius* in the absence of an effective amount of a supplemental saccharide. The improvement is observed whether the supplemental saccharide is added to a composition comprising other saccharides, or a composition which is saccharide free.

[00166] As used herein the term "upregulating a gene or genes" means increasing expression levels of the transcribed RNA encoded by the gene resulting in increased

peptide, bacteriocin or protein. The related term "a method of upregulating a gene" means a method of increasing expression levels of the transcribed RNA encoded by the gene resulting in increased peptide, bacteriocin or protein. In embodiments, the method results in increase of expression levels of the transcribed RNA encoded by the gene resulting in increased expression of said peptide, bacteriocin or protein, and/or increase of production of the said peptide, bacteriocin or protein.

[00167] As used herein "improving the mucoid properties" of an *S. salivarius* species means bacterial colonies of the *S. salivarius* are more mucoid (sticky mucus-like) making the probiotic more capable of adhering to substrates and thus allowing for enhanced colonisation.

[00168] An "ENTR" microorganism is an ear, nose, throat, or respiratory tract microorganism and includes pathogens that infect the ear (aural), nose (nasal), throat, upper respiratory tract, and lower respiratory tract, and non-pathogenic microorganisms that colonise the ear (aural), nose (nasal), throat, upper respiratory tract, and lower respiratory tract. The term "lower respiratory tract" or "LRT" as used herein means the trachea, bronchi, and lungs. The term is contrasted with the "upper respiratory tract" or "URT" which means nose, nasal passages, and nasopharynx.

[00169] A "salivaricin" is a bacteriocin-like inhibitory substance (BLIS) produced by a *S. salivarius* strain. Accordingly, a Blis-producing *S. salivarius* is an *S. salivarius* which produces a salivaricin.

[00170] The term "microbe" or "microorganism" as used herein refers to a bacteria, fungus, virus or a combination thereof. Similarly, the term "microbial" population, infection, disease or condition as used herein includes bacterial, viral and fungal populations, infections, diseases, or conditions. A microorganism may be pathogenic, or non-pathogenic (e.g. commensal).

[00171] The term an "effective amount" as used herein means an amount effective to inhibit, reduce or control a microbial population, or to protect against, delay, reduce, stabilise, improve or treat a microbial infection in and/or on a patient. It also refers to an amount sufficient to provide a beneficial effect to a patient. Such beneficial effects may include detectable: increase in IFN-γ, reduction in NF-kB-mediated cytokine response in lungs, inhibition of microbial replication, and/or decrease in microbial load. The effect should be sufficient to provide a medically significant decrease in the likelihood of a bacterial, fungal or viral infection, or a medically or statistically significant decrease in the

rate, extent, severity, or length of a microbial infection, or associated symptoms, or secondary infections. Reduction in the survival, growth and/or proliferation of the microorganism is contemplated.

[00172] In one embodiment the term "statistically significant" as used herein refers to the likelihood that a result or relationship is caused by something other than random chance. A result may be found to be statistically significant using statistical hypothesis testing as known and used in the art. Statistical hypothesis testing provides a "P-value" as known in the art, which represents the probability that the measured result is due to random chance alone. It is believed to be generally accepted in the art that levels of significance of 5% (0.05) or lower are considered to be statistically significant.

[00173] The phrase "inhibiting the growth of a microorganism sensitive to a composition of the invention" and like terms refer to the growth inhibition of at least one or more species of microorganism sensitive to a BLIS-producing streptococcal strain, e.g., *S. salivarius*. Inhibition of bacterial growth can be determined by a variety of methods including inhibition of colony forming units (CFU) of a targeted bacterial strain as described in WO01/27143. Inhibition of viral growth may be determined by a variety of methods but can include the Virus Yield Reduction Assay (VYR), observation of cytopathic effect or virucidal assays. Inhibition of fungal growth can be determined e.g. by adaption for fungi of the deferred antagonism method (Tagg and Bannister 1979; *Med Microbiology* 12:397) as described in the examples. Inhibition of germ tube formation provides another measure of anti-fungal activity (e.g. Reynolds and Braude (1956) *Clin Res Proc* 4:40).

[00174] The term "contacting" as used herein refers to both direct and indirect contact between the microorganism and a Blis composition. Indirect contact comprises exposure of the microorganism in its environment, particularly native environment, to a Blis composition.

[00175] A "composition" or "Blis composition" as used herein refers to a composition or formulation comprising *S. salivarius* K12, M18 or combination thereof, and may include BLIS-containing naturally-released extracellular products thereof such as salivaricins or other antimicrobial agents; and optionally a carrier, diluent or excipient. In some embodiments, the composition can be a cosmetic, a dietary supplement, a natural health product, or a complementary medicine. A dietary supplement (also known as a food supplement) is a foodstuff that is intended to supplement the normal diet and contains concentrated nutrients, that is vitamins or minerals, or other substances with a nutritional

or physiological effect. A natural health product includes probiotics, herbal remedies, vitamins and minerals, homeopathic medicines, traditional medicines, and amino acids and essential fatty acids. A complementary medicine is a medicine which has been evaluated for safety and quality, and may have been evaluated for efficacy.

[00176] The unit "cfu/g" means colony-forming units per gram.

[00177] As used herein, the term "variant" refers to gene, polynucleotide, or polypeptide sequences different from the specifically identified sequences, wherein one or more nucleotides or amino acid residues is deleted, substituted, or added. Variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variants may be from the same or from other species and may encompass homologues, paralogues and orthologues.

[00178] In certain embodiments, variants of the polynucleotides and polypeptides disclosed herein possess biological activities that are the same or similar to those of the polynucleotides or polypeptides disclosed herein. The term "variant" with reference to polynucleotides and polypeptides encompasses all forms of polynucleotides and polypeptides as defined herein.

Method of the invention

[00179] In a first aspect, the invention provides a method of improving the inhibitory profile of *S. salivarius* comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide, wherein the *Streptococcus salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

[00180] Potency of inhibition is the extent to which an inhibited microorganism is inhibited. Potency of inhibition may be measured by determining the size of the zone of inhibition (ZOI) compared to a control sample on an agar plate, as described in Tagg JR, Bannister LV. "Fingerprinting" beta-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. *J Med Microbiol.* **1979** Nov;12(4):397-411. A more potent inhibitory effect will be evident by a larger zone of inhibition. Another method of determining potency of inhibition is a liquid assay, such as is described in Enhanced Production, Purification, Characterization and Mechanism of Action of Salivaricin 9 Lantibiotic Produced by *Streptococcus salivarius* NU10, *PLoS One* (**2013**) 8(10): e77751. For viruses, potency can be measured through Selectivity Index (SI) that measures the ratio of the toxic concentration of a sample against its effective bioactive

concentration in a cell line experiment. Greater potency would be indicated with a higher SI index compared to a control. Other methods of determining potency of inhibition will be apparent to a skilled worker.

[00181] *S. salivarius* strains useful herein can be characterised at least in part by deferred antagonism assay (P-typing - inhibition of indicator strains), or determining which salivaricins the strain produces. This is described in Tagg and Bannister (1979) *J. Med. Microbiol.* 12:397. K12 display a P-type 777 (see WO2001027143) and M18 display a P-type 677 on Blood agar with calcium carbonate, and a P-type 777 on Trypticase soy-yeast extract-calcium carbonate agar (see WO2003070919).

[00182] The spectrum of inhibitory activity is the number of inhibited microorganisms. In various embodiments, the *S. salivarius* of the invention inhibits a microorganism or species that is not inhibited by the *S. salivarius* in the absence of an effective amount of a supplemental saccharide. The applicant has unexpectedly found that supplemental saccharides can not only impact growth and inhibitory activity of *S. salivarius* probiotics but can also increase the range of microorganisms against which the probiotic is active. This is believed to be the first time that a change in the spectrum of activity for such probiotics has been reported. As described above, inhibitory activity may be measured by determining the size of the zone of inhibition compared to a control sample on an agar plate. A species that is not inhibited will have a zone of inhibition of zero or an SI index of less than 4.9.

[00183] In one aspect, the invention provides a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide for use in improving the inhibitory profile of the *S. salivarius*, wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00184] Described herein is a method of inhibiting a non-pathogenic bacteria, the method comprising contacting the bacteria with a composition comprising *S.salivarius* and an effective amount of a supplemental saccharide, wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and wherein the supplemental saccharide is galactose or raffinose or a combination thereof. In various embodiments, the bacteria are selected from *L. lactis*, *S. epidermidis*, and *S. constellatus*.

[00185] As discussed in the background, simple sugars are known to promote the growth of bacteria by being consumed as an energy source and produce organic acid by-

products, which may have a weak non-selective inhibitory effect on the growth of other bacteria. Surprisingly, the inventors have demonstrated that the improved inhibitory profile of *S. salivarius* is not solely due to pH effects of the supplementary saccharide (see Examples 4 and 6). Without being bound by theory, the inventors believe the improved inhibitory profile is due to enhanced activity of bacteriocin(s) or other encoded antimicrobial(s) (e.g. non-ribosomal peptide-synthetase (NRPS)). Additionally, the inventors have shown that raffinose unexpectedly influences the morphology of cells resulting in larger sized and more mucoid (sticky mucus-like) colonies making the probiotic more capable of adhering to substrates and thus allowing for enhanced colonisation (see Example 11).

Streptococcus salivarius

[00186] *S. salivarius* is a gram-positive bacterium that predominantly colonises the human oral cavity and are the dominant commensal species. They are highly investigated for use as probiotic bacteria. Two *S. salivarius* strains have been commercialised by Blis Technologies Ltd with trade names BLIS M18™ and BLIS K12™ for oral and dental health.

[00187] A range of *S. salivarius* strains used in the methods of the invention are known in the art. *S. salivarius* K12 was deposited with Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124, Braunschweig, Germany on 8 October 1999, and assigned Accession Nos. DSM 13084. *S. salivarius* M18 was deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124, Braunschweig, Germany on 12 December 2001, and assigned Accession No. DSM 14685. *S. salivarius* M18 is described in WO20030709191, incorporated herein by reference.

[00188] Bacteriocins are antagonistic substances which have evolved as a conserved biological tool capable of conferring a competitive advantage to the productive organism. Bacteriocins are ribosomally-synthesised antimicrobial peptides produced by bacteria to inhibit the growth of closely related species. A diverse range of bacteriocins have been identified and categorised into four main classes, which are further divided into sub-classes. Bacteriocins classes are determined by parameters such as the productive species, the mechanism action, the spectrum of activity, and the degree of post-translational modification.

[00189] BLIS K12™ produces bacteriocins belonging to Class I and Class II: salivaricin A (SEQ ID NOs 15 and 23), salivaricin B (SEQ ID NOs 16 and 24) and salivaricin Q (SEQ ID NOs 17 and 25) (*salA*, *salB* and *salQ* respectively). BLIS M18™ produces bacteriocins belonging to Class I and Class II: salivaricin A (SEQ ID NOs 19 and 27), salivaricin 9 (SEQ ID NOs 20 and 28) and salivaricin Q (SEQ ID NOs 21 and 29) (*salA*, *sal9* and *salQ* respectively). SalA and SalB are post-translationally modified peptide molecules which display high target specificity for gram-positive, group-A streptococci (i.e. *Streptococcus pyogenes*). SalA functions by binding to target membrane structures and forming pores in the structure to disrupt the structural integrity of the membrane ultimately leading to cell death. SalB functions by inhibiting target species' enzymatic processes which leads to the prevention of key cellular processes resulting in cell death. A bacteriocin with greater than 90% identity to *salQ* has also been characterized as a "*blpU-like cassette*" (Santagati *et al., Front. Biosci. (Schol Ed)* **2018**, 10(2), 238–247.) BLIS K12™ and BLIS M18™ also produce distinct versions of salivaricin MPS which is a Class III bacteriocin. Class III bacteriocins are large, unmodified, heat-labile peptide molecules which target conserved structures expressed by gram-positive streptococci and other bacterial species. BLIS K12™ also produces the antimicrobial NRPS.

[00190] The urease protein is important for modulating pH homeostasis for bacteria and when bacteria produce it in the oral cavity it has the effect of modulating the pH of mouth and saliva to less acidic and more towards neutral pH which is of benefit to dental health. Ure C is the alpha subunit of the urease protein encoded by the *ureC* gene. While the functional protein comprises more subunits, expression of the protein can be inferred based on the *ureC* gene for BLIS K12™ (SEQ ID NO 18) and BLIS M18™ (SEQ ID NO 22).

[00191] In various embodiments, compositions useful in the invention comprise at least 0.1wt%. of *S. salivarius*.

[00192] In various embodiments, the composition comprises from about 0.1 to about 20wt% of *S. salivarius*, for example from about 0.1 to about 18wt%, about 0.1 to about 16wt%, about 0.1 to about 15wt%, about 0.1 to about 14wt%, about 0.1 to about 12wt%, about 0.1 to about 10wt%, about 0.1 to about 9wt%, about 0.1 to about 8wt%, about 0.1 to about 7wt%, about 0.1 to about 6 wt%, about 0.1 to about 5 wt%, about 0.5 to about 18wt%, about 0.5 to about 16wt%, about 0.5 to about 15wt%, about 0.5 to about 14wt%, about 0.5 to about 12wt%, about 0.5 to about 10wt%, about 0.5 to about 9wt%, about 0.5 to about 8wt%, about 0.5 to about 7wt%, about 0.5 to about 6 wt%, about 0.5 to about 5 wt%, about 1 to about 18wt%, about 1 to about 16wt%, about 1 to about 15wt%, about 1

to about 14wt%, about 1 to about 12wt%, about 1 to about 10wt%, about 1 to about 9wt%, about 1 to about 8wt%, about 1 to about 7wt%, about 1 to about 6 wt%, or about 1 to about 5 wt% of *S. salivarius*.

[00193] In various embodiments, the composition comprises the *S. salivarius* probiotic cells in from about 1×10^3 to about 1×10^{13} cfu/g. In various embodiments, the composition comprises *S. salivarius* cells in an amount of about 1×10^4 to about 1×10^{12}, about 1×10^5 to about 1×10^{12}, about 1×10^6 to about 1×10^{12}, about 1×10^7 to about 1×10^{12}, about 1×10^8 to about 1×10^{12}, about 1×10^4 to about 1×10^{10}, about 1×10^5 to about 1×10^{10}, about 1×10^6 to about 1×10^{10}, about 1×10^7 to about 1×10^{10}, about 1×10^8 to about 1×10^{10}, about 1×10^4 to about 1×10^9, about 1×10^5 to about 1×10^9, about 1×10^6 to about 1×10^9, about 1×10^7 to about 1×10^9 cfu/g. In various embodiments, the product or composition comprises *S. salivarius* cells in an amount of about 1×10^9 cfu/g.

[00194] In various embodiments, where multiple strains of *S. salivarius* are present, the composition comprises at least 0.1 wt% of each strain of *S. salivarius*.

[00195] In various embodiments, where multiple strains of *S. salivarius* are present, the composition comprises from about 0.1 to about 20wt% of each strain of *S. salivarius*, for example from about 0.1 to about 18wt%, about 0.1 to about 16wt%, about 0.1 to about 15wt%, about 0.1 to about 14wt%, about 0.1 to about 12wt%, about 0.1 to about 10wt%, about 0.1 to about 9wt%, about 0.1 to about 8wt%, about 0.1 to about 7wt%, about 0.1 to about 6 wt%, about 0.1 to about 5 wt%, about 0.5 to about 18wt%, about 0.5 to about 16wt%, about 0.5 to about 15wt%, about 0.5 to about 14wt%, about 0.5 to about 12wt%, about 0.5 to about 10wt%, about 0.5 to about 9wt%, about 0.5 to about 8wt%, about 0.5 to about 7wt%, about 0.5 to about 6 wt%, about 0.5 to about 5 wt%, about 1 to about 18wt%, about 1 to about 16wt%, about 1 to about 15wt%, about 1 to about 14wt%, about 1 to about 12wt%, about 1 to about 10wt%, about 1 to about 9wt%, about 1 to about 8wt%, about 1 to about 7wt%, about 1 to about 6 wt%, or about 1 to about 5 wt% of each strain of *S. salivarius*.

[00196] In various embodiments, where multiple strains of *S. salivarius* are present, the composition comprises from about 1×10^3 to about 1×10^{13} cfu/g of each strain of *S. salivarius*, for example from about 1×10^4 to about 1×10^{12}, about 1×10^5 to about 1×10^{12}, about 1×10^6 to about 1×10^{12}, about 1×10^7 to about 1×10^{12}, about 1×10^8 to about 1×10^{12}, about 1×10^4 to about 1×10^{10}, about 1×10^5 to about 1×10^{10}, about 1×10^6 to about 1×10^{10}, about 1×10^7 to about 1×10^{10}, about 1×10^8 to about 1×10^{10}, about 1×10^4 to about 1×10^9,

about 1×10^5 to about 1×10^9, about 1×10^6 to about 1×10^9, about 1×10^7 to about 1×10^9 cfu/g of each strain of *S. salivarius*.

Supplemental saccharide

[00197] Saccharides considered for testing included the monosaccharides glucose, fructose and galactose; the disaccharides sucrose and lactose; and the trisaccharide raffinose.

[00198] Lactose is primarily a dairy sugar and is not a preferred ingredient for people on dairy-free diets or people with lactose intolerance. Lactose was therefore excluded from consideration.

[00199] As discussed above, sucrose and glucose are cariogenic and generally regarded as having a suppressive effect on antagonistic behavior (see, for example, Reinhold & Titgemeyer, Fritz. (**2002**). *FEMS Microbiology Letters*. 209. 141-8; Jankovic & Brückner, *J Mol Microbiol Biotechnol*. **2007**;12(1-2):114-20). These saccharides were therefore also excluded from consideration.

[00200] In various embodiments, the supplemental saccharide is galactose, or raffinose, or a combination thereof. In various embodiments, the supplemental saccharide is galactose. In various embodiments, the supplemental saccharide is raffinose. In various embodiments, the supplemental saccharide is a mixture of galactose and raffinose.

[00201] D-Galactose is a monosaccharide sugar. Raffinose is a trisaccharide comprising galactose, glucose, and fructose monomer units.

[00202] In various embodiments, the composition comprises less than 20% by weight of each supplemental saccharide. In various embodiments, the composition comprises less than 20% by weight of galactose. In various embodiments, the composition comprises less than 20% by weight of raffinose. In various embodiments, the composition comprises less than 20% by weight of each of galactose and raffinose.

[00203] In various embodiments, the composition comprises 0.01-20% by weight of each supplemental saccharide, for example from about 0.01 to about 18%, or about 0.01 to about 15%, or about 0.01 to about 12%, or about 0.01 to about 10%, or about 0.01 to about 9%, or about 0.01 to about 8%, or about 0.01 to about 7%, or about 0.01 to about 6%, or about 0.01 to about 5%, or about 0.01 to about 4%, or about 0.1 to about 20%, or about 0.1 to about 18%, or about 0.1 to about 15%, or about 0.1 to about 12%, or about 0.1 to about

10%, or about 0.1 to about 9%, or about 0.1 to about 8%, or about 0.1 to about 7%, or about 0.1 to about 6%, or about 0.1 to about 5%, or about 0.1 to about 4%, or about 0.25 to about 20%, or about 0.25 to about 18%, or about 0.25 to about 15%, or about 0.25 to about 12%, or about 0.25 to about 10%, or about 0.25 to about 9%, or about 0.25 to about 8%, or about 0.25 to about 7%, or about 0.25 to about 6%, or about 0.25 to about 5%, or about 0.25 to about 4%, or about 0.5 to about 20%, or about 0.5 to about 18%, or about 0.5 to about 15%, or about 0.5 to about 12%, or about 0.5 to about 10%, or about 0.5 to about 9%, or about 0.5 to about 8%, or about 0.5 to about 7%, or about 0.5 to about 6%, or about 0.5 to about 5%, or about 0.5 to about 4%, or about 1 to about 20%, or about 1 to about 18%, or about 1 to about 15%, or about 1 to about 12%, or about 1 to about 10%, or about 1 to about 9%, or about 1 to about 8%, or about 1 to about 7%, or about 1 to about 6%, or about 1 to about 5%, or about 1 to about 4%, or about 1.25 to about 20%, or about 1.25 to about 18%, or about 1.25 to about 15%, or about 1.25 to about 12%, or about 1.25 to about 10%, or about 1.25 to about 9%, or about 1.25 to about 8%, or about 1.25 to about 7%, or about 1.25 to about 6%, or about 1.25 to about 5%, or about 1.25 to about 4%, or about 2 to about 20%, or about 2 to about 18%, or about 2 to about 15%, or about 2 to about 12%, or about 2 to about 10%, or about 2 to about 9%, or about 2 to about 8%, or about 2 to about 7%, or about 2 to about 6%, or about 2 to about 5%, or about 2 to about 4%, or about 2.5 to about 20%, or about 2.5 to about 18%, or about 2.5 to about 15%, or about 2.5 to about 12%, or about 2.5 to about 10%, or about 2.5 to about 9%, or about 2.5 to about 8%, or about 2.5 to about 7%, or about 2.5 to about 6%, or about 2.5 to about 5%, or about 2.5 to about 4% by weight of each supplemental saccharide.

[00204] In various embodiments, the composition comprises 0.01-20% by weight of galactose, for example from about 0.01 to about 18%, or about 0.01 to about 15%, or about 0.01 to about 12%, or about 0.01 to about 10%, or about 0.01 to about 9%, or about 0.01 to about 8%, or about 0.01 to about 7%, or about 0.01 to about 6%, or about 0.01 to about 5%, or about 0.01 to about 4%, or about 0.1 to about 20%, or about 0.1 to about 18%, or about 0.1 to about 15%, or about 0.1 to about 12%, or about 0.1 to about 10%, or about 0.1 to about 9%, or about 0.1 to about 8%, or about 0.1 to about 7%, or about 0.1 to about 6%, or about 0.1 to about 5%, or about 0.1 to about 4%, or about 0.25 to about 20%, or about 0.25 to about 18%, or about 0.25 to about 15%, or about 0.25 to about 12%, or about 0.25 to about 10%, or about 0.25 to about 9%, or about 0.25 to about 8%, or about 0.25 to about 7%, or about 0.25 to about 6%, or about 0.25 to about 5%, or about 0.25 to about 4%, or about 0.5 to about 20%, or about 0.5 to about 18%, or about 0.5 to about 15%, or about 0.5 to about 12%, or about 0.5 to about 10%, or about 0.5 to about 9%, or about 0.5 to about 8%, or about 0.5 to about 7%, or about 0.5 to about 6%, or about 0.5 to

about 5%, or about 0.5 to about 4%, or about 1 to about 20%, or about 1 to about 18%, or about 1 to about 15%, or about 1 to about 12%, or about 1 to about 10%, or about 1 to about 9%, or about 1 to about 8%, or about 1 to about 7%, or about 1 to about 6%, or about 1 to about 5%, or about 1 to about 4%, or about 1.25 to about 20%, or about 1.25 to about 18%, or about 1.25 to about 15%, or about 1.25 to about 12%, or about 1.25 to about 10%, or about 1.25 to about 9%, or about 1.25 to about 8%, or about 1.25 to about 7%, or about 1.25 to about 6%, or about 1.25 to about 5%, or about 1.25 to about 4%, or about 2 to about 20%, or about 2 to about 18%, or about 2 to about 15%, or about 2 to about 12%, or about 2 to about 10%, or about 2 to about 9%, or about 2 to about 8%, or about 2 to about 7%, or about 2 to about 6%, or about 2 to about 5%, or about 2 to about 4%, or about 2.5 to about 20%, or about 2.5 to about 18%, or about 2.5 to about 15%, or about 2.5 to about 12%, or about 2.5 to about 10%, or about 2.5 to about 9%, or about 2.5 to about 8%, or about 2.5 to about 7%, or about 2.5 to about 6%, or about 2.5 to about 5%, or about 2.5 to about 4% by weight of galactose.

[00205] In various embodiments, the composition comprises 0.01-20% by weight of raffinose, for example from about 0.01 to about 18%, or about 0.01 to about 15%, or about 0.01 to about 12%, or about 0.01 to about 10%, or about 0.01 to about 9%, or about 0.01 to about 8%, or about 0.01 to about 7%, or about 0.01 to about 6%, or about 0.01 to about 5%, or about 0.01 to about 4%, or about 0.1 to about 20%, or about 0.1 to about 18%, or about 0.1 to about 15%, or about 0.1 to about 12%, or about 0.1 to about 10%, or about 0.1 to about 9%, or about 0.1 to about 8%, or about 0.1 to about 7%, or about 0.1 to about 6%, or about 0.1 to about 5%, or about 0.1 to about 4%, or about 0.25 to about 20%, or about 0.25 to about 18%, or about 0.25 to about 15%, or about 0.25 to about 12%, or about 0.25 to about 10%, or about 0.25 to about 9%, or about 0.25 to about 8%, or about 0.25 to about 7%, or about 0.25 to about 6%, or about 0.25 to about 5%, or about 0.25 to about 4%, or about 0.5 to about 20%, or about 0.5 to about 18%, or about 0.5 to about 15%, or about 0.5 to about 12%, or about 0.5 to about 10%, or about 0.5 to about 9%, or about 0.5 to about 8%, or about 0.5 to about 7%, or about 0.5 to about 6%, or about 0.5 to about 5%, or about 0.5 to about 4%, or about 1 to about 20%, or about 1 to about 18%, or about 1 to about 15%, or about 1 to about 12%, or about 1 to about 10%, or about 1 to about 9%, or about 1 to about 8%, or about 1 to about 7%, or about 1 to about 6%, or about 1 to about 5%, or about 1 to about 4%, or about 1.25 to about 20%, or about 1.25 to about 18%, or about 1.25 to about 15%, or about 1.25 to about 12%, or about 1.25 to about 10%, or about 1.25 to about 9%, or about 1.25 to about 8%, or about 1.25 to about 7%, or about 1.25 to about 6%, or about 1.25 to about 5%, or about 1.25 to about 4%, or about 2 to about 20%, or about 2 to about 18%, or about 2 to about 15%, or about 2 to about 12%, or about 2 to

about 10%, or about 2 to about 9%, or about 2 to about 8%, or about 2 to about 7%, or about 2 to about 6%, or about 2 to about 5%, or about 2 to about 4%, or about 2.5 to about 20%, or about 2.5 to about 18%, or about 2.5 to about 15%, or about 2.5 to about 12%, or about 2.5 to about 10%, or about 2.5 to about 9%, or about 2.5 to about 8%, or about 2.5 to about 7%, or about 2.5 to about 6%, or about 2.5 to about 5%, or about 2.5 to about 4% by weight of raffinose.

[00206] In various embodiments, where a mixture of galactose and raffinose is present, the composition comprises 0.01-20% by weight of each of galactose and raffinose, for example from about 0.01 to about 18%, or about 0.01 to about 15%, or about 0.01 to about 12%, or about 0.01 to about 10%, or about 0.01 to about 9%, or about 0.01 to about 8%, or about 0.01 to about 7%, or about 0.01 to about 6%, or about 0.01 to about 5%, or about 0.01 to about 4%, or about 0.1 to about 20%, or about 0.1 to about 18%, or about 0.1 to about 15%, or about 0.1 to about 12%, or about 0.1 to about 10%, or about 0.1 to about 9%, or about 0.1 to about 8%, or about 0.1 to about 7%, or about 0.1 to about 6%, or about 0.1 to about 5%, or about 0.1 to about 4%, or about 0.25 to about 20%, or about 0.25 to about 18%, or about 0.25 to about 15%, or about 0.25 to about 12%, or about 0.25 to about 10%, or about 0.25 to about 9%, or about 0.25 to about 8%, or about 0.25 to about 7%, or about 0.25 to about 6%, or about 0.25 to about 5%, or about 0.25 to about 4%, or about 0.5 to about 20%, or about 0.5 to about 18%, or about 0.5 to about 15%, or about 0.5 to about 12%, or about 0.5 to about 10%, or about 0.5 to about 9%, or about 0.5 to about 8%, or about 0.5 to about 7%, or about 0.5 to about 6%, or about 0.5 to about 5%, or about 0.5 to about 4%, or about 1 to about 20%, or about 1 to about 18%, or about 1 to about 15%, or about 1 to about 12%, or about 1 to about 10%, or about 1 to about 9%, or about 1 to about 8%, or about 1 to about 7%, or about 1 to about 6%, or about 1 to about 5%, or about 1 to about 4%, or about 1.25 to about 20%, or about 1.25 to about 18%, or about 1.25 to about 15%, or about 1.25 to about 12%, or about 1.25 to about 10%, or about 1.25 to about 9%, or about 1.25 to about 8%, or about 1.25 to about 7%, or about 1.25 to about 6%, or about 1.25 to about 5%, or about 1.25 to about 4%, or about 2 to about 20%, or about 2 to about 18%, or about 2 to about 15%, or about 2 to about 12%, or about 2 to about 10%, or about 2 to about 9%, or about 2 to about 8%, or about 2 to about 7%, or about 2 to about 6%, or about 2 to about 5%, or about 2 to about 4%, or about 2.5 to about 20%, or about 2.5 to about 18%, or about 2.5 to about 15%, or about 2.5 to about 12%, or about 2.5 to about 10%, or about 2.5 to about 9%, or about 2.5 to about 8%, or about 2.5 to about 7%, or about 2.5 to about 6%, or about 2.5 to about 5%, or about 2.5 to about 4% of each of galactose and raffinose.

Upregulated genes

[00207] In one aspect, the invention provides a method for upregulating one or more genes in *Streptococcus salivarius*, comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

[00208] In some embodiments, the upregulated gene(s) may comprise or consist of a polynucleotide sequence that encodes a lantibiotic peptide or a bacteriocin, such as a Class I or Class II lantibiotic peptide or bacteiocin. For example, as described above, BLIS K12™ produces the bacteriocins salivaricin A, salivaricin B, and salivaricin Q, encoded by *salA*, *salB* and *salQ* respectively (SEQ ID NOs 15, 16, and 17 respectively). The peptide sequences of these bacteriocins are presented in SEQ ID NOs 23, 24, and 25, respectively.

[00209] As a further example, as described above, BLIS M18™ produces the bacteriocins salivaricin A, salivaricin 9, and salivaricin Q, encoded by *salA*, *sal9* and *salQ* respectively (SEQ ID NOs 19, 20, and 21 respectively). The peptide sequences of these bacteriocins are presented in SEQ ID NOs 27, 28, and 29 respectively.

[00210] In some embodiments, the upregulated gene(s) may comprise or consist of a polynucleotide sequence that encodes a subunit of a urease protein. In some embodiments, the upregulated gene is ureC, for example, the BLIS K12™ ureC (SEQ ID NO 18) or the BLIS M18™ ureC (SEQ ID NO 22).

[00211] In some embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 70% sequence identity to any one of SEQ ID NOs 15-22, or at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 70% sequence identity to any one of SEQ ID NOs 23-30.

[00212] In some embodiments, at least one of the upregulated gene(s) is a *sal*A gene, a *sal*B gene, a *sal*Q gene, a *sal*9 gene, and/or a *ure*C gene, or a variant of any of these.

[00213] These genes and variants thereof can be readily identified by techniques known in the art. For example, these genes may be identified by sequence similarity to

known salA, salB, salQ, sal9, or ureC genes, or to genes encoding salA, salB, salQ, sal9, or ureC proteins, using sequence alignment tools. Alternatively, genes encoding proteins may be identified by structural similarity to known salA, salB, salQ, sal9, or ureC proteins using structural alignment.

[00214] For example, these genes may be identified by computer-based methods well-known to those skilled in the art, using public domain sequence alignment algorithms and sequence similarity search tools to search sequence databases (public domain databases include Genbank, EMBL, Swiss-Prot, PIR and others). See, e.g., Nucleic Acids Res. 29: 1-10 and 11-16, 2001 for examples of online resources. Similarity searches retrieve and align target sequences for comparison with a sequence to be analyzed (i.e., a query sequence). Sequence comparison algorithms use scoring matrices to assign an overall score to each of the alignments.

[00215] An exemplary family of programs useful for identifying variants in sequence databases is the BLAST suite of programs (version 2.2.5 [Nov 2002]) including BLASTN, BLASTP, BLASTX, tBLASTN and tBLASTX, which are publicly available from (ftp://ftp.ncbi.nih.gov/blast/) or from the National Center for Biotechnology Information (NCBI), National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894 USA. The use of the BLAST family of algorithms, including BLASTN, BLASTP, and BLASTX, is described in the publication of Altschul *et al.*, Nucleic Acids Res. 25: 3389-3402, 1997.

[00216] Multiple sequence alignments of a group of related sequences can be carried out with CLUSTALW (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680) CLUSTAL Omega (Sievers et al., (2011). Molecular Systems Biology 7:539, https://www.ebi.ac.uk/Tools/msa/clustalo/) or T-COFFEE (Cedric Notredame, Desmond G. Higgins, Jaap Heringa, T-Coffee: A novel method for fast and accurate multiple sequence alignment, J. Mol. Biol. (2000) 302: 205-217)) or PILEUP, which uses progressive, pairwise alignments. (Feng and Doolittle, 1987, J. Mol. Evol. 25, 351).

[00217] Pattern recognition software applications are available for finding motifs or signature sequences. For example, MEME (Multiple Em for Motif Elicitation) finds motifs and signature sequences in a set of sequences, and MAST (Motif Alignment and Search Tool) uses these motifs to identify similar or the same motifs in query sequences. The

MAST results are provided as a series of alignments with appropriate statistical data and a visual overview of the motifs found. MEME and MAST were developed at the University of California, San Diego.

[00218] PROSITE (Bairoch and Bucher, 1994, Nucleic Acids Res. 22, 3583; Hofmann *et al.*, 1999, Nucleic Acids Res. 27, 215) is a method of identifying the functions of uncharacterized proteins translated from genomic or cDNA sequences. The PROSITE database (www.expasy.org/prosite) contains biologically significant patterns and profiles and is designed so that it can be used with appropriate computational tools to assign a new sequence to a known family of proteins or to determine which known domain(s) are present in the sequence (Falquet *et al.*, 2002, Nucleic Acids Res. 30, 235). Prosearch is a tool that can search SWISS-PROT and EMBL databases with a given sequence pattern or signature.

[00219] Another example of a protein domain model database is Pfam (Sonnhammer et al., 1997, A comprehensive database of protein families based on seed alignments, Proteins, 28: 405-420; Finn et al., 2010, The Pfam protein families database', Nucl. Acids Res., 38: D211–D222). "Pfam" refers to a large collection of protein domains and protein families maintained by the Pfam Consortium and available at several sponsored world wide web sites, including: pfam.xfam.org/ (European Bioinformatics Institute (EMBL-EBI). The latest release of Pfam is Pfam 35.0 (November 2021). Pfam domains and families are identified using multiple sequence alignments and hidden Markov models (HMMs). Pfam-A family or domain assignments, are high quality assignments generated by a curated seed alignment using representative members of a protein family and profile hidden Markov models based on the seed alignment. (Unless otherwise specified, matches of a queried protein to a Pfam domain or family are Pfam-A matches.) All identified sequences belonging to the family are then used to automatically generate a full alignment for the family (Sonnhammer (1998) Nucleic Acids Research 26, 320-322; Bateman (2000) Nucleic Acids Research 26, 263-266; Bateman (2004) Nucleic Acids Research 32, Database Issue, D138-D141; Finn (2006) Nucleic Acids Research Database Issue 34, D247-251; Finn (2010) Nucleic Acids Research Database Issue 38, D21 1-222). By accessing the Pfam database, for example, using the above-referenced website, protein sequences can be queried against the HMMs using HMMER homology search software {e.g., HMMER2, HMMER3, or a higher version, hmmer.org). Significant matches that identify a queried protein as being in a pfam family (or as having a particular Pfam domain) are those in which the bit score is greater than or equal to the gathering threshold for the Pfam domain. Expectation values (e values) can also be used as a criterion for

inclusion of a queried protein in a Pfam or for determining whether a queried protein has a particular Pfam domain, where low e values (much less than 1.0, for example less than 0.1, or less than or equal to 0.01) represent low probabilities that a match is due to chance.

[00220] Some specific examples of known genes from *S. salivarius* are provided herein according to the following table.

Strain	Gene name	DNA sequence	Protein sequence
K12	SalA	SEQ ID NO 15	SEQ ID NO 23
K12	SalB	SEQ ID NO 16	SEQ ID NO 24
K12	SalQ	SEQ ID NO 17	SEQ ID NO 25
K12	UreC	SEQ ID NO 18	SEQ ID NO 26
M18	SalA	SEQ ID NO 19	SEQ ID NO 27
M18	Sal9	SEQ ID NO 20	SEQ ID NO 28
M18	SalQ	SEQ ID NO 21	SEQ ID NO 29
M18	UreC	SEQ ID NO 22	SEQ ID NO 30

[00221] In some embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 70% identity to any one of SEQ ID Nos 15-22, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00222] In some embodiments, at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 70% identity to any one of SEQ ID Nos 23-30, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00223] In some embodiments, at least one of the upregulated gene(s) is a salA gene that comprises or consists of a polynucleotide sequence with at least 70% identity to SEQ ID NO 15 or 19, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity; or that encodes a polypeptide with at least 70% identity to SEQ ID NO 23 or 27, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00224] In some embodiments, at least one of the upregulated gene(s) is a salB gene that comprises or consists of a polynucleotide sequence with at least 70% identity to SEQ ID NO 16, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity; or that encodes a polypeptide with at least 70% identity to SEQ ID NO 24, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00225] In some embodiments, at least one of the upregulated gene(s) is a salQ gene that comprises or consists of a polynucleotide sequence with at least 70% identity to SEQ ID NO 17 or 21, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity; or that encodes a polypeptide with at least 70% identity to SEQ ID NO 25 or 29, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00226] In some embodiments, at least one of the upregulated gene(s) is a sal9 gene that comprises or consists of a polynucleotide sequence with at least 70% identity to SEQ ID NO 20, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity; or that encodes a polypeptide with at least 70% identity to SEQ ID NO 28, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

[00227] In some embodiments, at least one of the upregulated gene(s) is a ureC gene that comprises or consists of a polynucleotide sequence with at least 70% identity to SEQ ID NO 18 or 22, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity; or that encodes a polypeptide with at least 70% identity to SEQ ID NO 26 or 30, preferably at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity.

Determining polynucleotide sequence identity

[00228] Polynucleotide sequence identity can be determined in the following manner. The subject polynucleotide sequence is compared to a candidate polynucleotide sequence using BLASTN (from the BLAST suite of programs, version 2.2.5 [Nov 2002]) in bl2seq (Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250), which is publicly available from NCBI (ftp://ftp.ncbi.nih.gov/blast/). The default parameters of bl2seq are utilized except that filtering of low complexity parts should be turned off.

[00229] The identity of polynucleotide sequences may be examined using the following unix command line parameters:

[00230] bl2seq –i nucleotideseq1 –j nucleotideseq2 –F F –p blastn

[00231] The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. The bl2seq program reports sequence identity as both the number and percentage of identical nucleotides in a line “Identities = “.

[00232] Polynucleotide sequence identity may also be calculated over the entire length of the overlap between a candidate and subject polynucleotide sequences using global sequence alignment programs (e.g. Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). A full implementation of the Needleman-Wunsch global alignment algorithm is found in the needle program in the EMBOSS package (Rice,P. Longden,I. and Bleasby,A. EMBOSS: The European Molecular Biology Open Software Suite, Trends in Genetics June 2000, vol 16, No 6. pp.276-277) which can be obtained from http://www.hgmp.mrc.ac.uk/Software/EMBOSS/. The European Bioinformatics Institute server also provides the facility to perform EMBOSS-needle global alignments between two sequences online at http:/www.ebi.ac.uk/emboss/align/.

[00233] Alternatively the GAP program may be used which computes an optimal global alignment of two sequences without penalizing terminal gaps. GAP is described in the following paper: Huang, X. (1994) On Global Sequence Alignment. Computer Applications in the Biosciences 10, 227-235.

[00234] Another method for calculating polynucleotide % sequence identity is based on aligning sequences to be compared using Clustal X (Jeanmougin *et al.*, 1998, *Trends Biochem. Sci.* **23,** 403-5.)

[00235] Variant polynucleotides or genes as disclosed herein also encompass polynucleotides or genes that differ from the sequences as herein disclosed but that, as a consequence of the degeneracy of the genetic code, encode a polypeptide having similar activity to a polypeptide encoded by a polynucleotide disclosed herein. A sequence alteration that does not change the amino acid sequence of the polypeptide is a "silent variation".

[00236] Variant polynucleotides or genes as disclosed herein may comprise sequence alterations resulting in conservative substitutions of one or several amino acids in the encoded polypeptide sequence without significantly altering its biological activity.

[00237] Variant polynucleotides due to silent variations and conservative substitutions in the encoded polypeptide sequence may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov 2002]) from NCBI (ftp://ftp.ncbi.nih.gov/blast/) via the tblastx algorithm as previously described.

Determining polypeptide sequence identity

[00238] Polypeptide sequence identity can be determined in the following manner. The subject polypeptide sequence is compared to a candidate polypeptide sequence using BLASTP (from the BLAST suite of programs, version 2.2.5 [Nov 2002]) in bl2seq, which is publicly available from NCBI (ftp://ftp.ncbi.nih.gov/blast/). The default parameters of bl2seq are utilized except that filtering of low complexity regions should be turned off.

[00239] Polypeptide sequence identity may also be calculated over the entire length of the overlap between a candidate and subject polypeptide sequences using global sequence alignment programs. EMBOSS-needle (available at http:/www.ebi.ac.uk/emboss/align/) and GAP (Huang, X. (1994) On Global Sequence Alignment. Computer Applications in the Biosciences 10, 227-235.) as discussed above are also suitable global sequence alignment programs for calculating polypeptide sequence identity.

[00240] Another method for calculating polypeptide % sequence identity is based on aligning sequences to be compared using Clustal X (Jeanmougin *et al.*, 1998, *Trends Biochem. Sci.* **23,** 403-5.)

[00241] Polypeptide variants as disclosed herein also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides

may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov 2002]) from NCBI (ftp://ftp.ncbi.nih.gov/blast/). The similarity of polypeptide sequences may be examined using the following unix command line parameters:

[00242] bl2seq –i peptideseq1 –j peptideseq2 -F F –p blastp

[00243] The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. This program finds regions of similarity between the sequences and for each such region reports an "E value" which is the expected number of times one could expect to see such a match by chance in a database of a fixed reference size containing random sequences. For small E values, much less than one, this is approximately the probability of such a random match.

[00244] Variant polypeptide sequences preferably exhibit an E value of less than 1×10^{-10} more preferably less than 1×10^{-20}, more preferably less than 1×10^{-30}, more preferably less than 1×10^{-40}, more preferably less than 1×10^{-50}, more preferably less than 1×10^{-60}, more preferably less than 1×10^{-70}, more preferably less than 1×10^{-80}, more preferably less than 1×10^{-90} and most preferably less than 1×10^{-100} when compared with any one of the specifically identified sequences.

[00245] Variant polypeptide sequences may comprise conservative substitutions of one or several amino acids of a described polypeptide sequence without significantly altering its biological activity.

[00246] Identity is found over a comparison window of at least 20 amino acid positions, preferably at least 50 amino acid positions, more preferably at least 100 amino acid positions, and most preferably over the entire length of a polypeptide as herein disclosed.

Inhibited bacteria

[00247] In various embodiments, the method increases the inhibitory profile of *S. salivarius* against skin, oral, dental, mucosal (e.g. oral, rectal, vaginal) and/or ENTR microorganisms including pathogenic and other non-pathogenic microorganisms.

[00248] Microorganisms may be non-pathogenic. Subjects have an existing microflora which is generally not harmful, or may be beneficial to the subject. Examples

of such non-pathogenic microflora bacteria include *S. salivarius* spp., for example, *S. epidermidis* spp., *L. lactis* spp., and *S. constellatus* spp.

[00249] In some embodiments it may be useful to inhibit or reduce the population of such non-pathogenic microorganisms. For example, to facilitate colonisation with a Blis-producing *S. salivarius* such as K12 or M18.

[00250] In various embodiments, the skin, oral, dental, mucosal, and/or ENT microorganism is selected from *S. aureus* spp., *S. intermedius spp.*, *S. saprophyticus* spp., *M. catarrhalis* spp., *H. influenzae* spp., *S. pyogenes* spp., *P. aeruginosa* spp., *S. mutans* spp., *S. pneumoniae* spp., *C. acnes* spp., *C. albicans* spp. *S. sobrin*us spp., *Corynebacterium* spp., *F. nucleatum* spp., *A. actinomycetemcomitans* spp., *P. gingivalis* spp., *Tannerella forsythia* spp., *Treponema denticola* spp., *P. intermedia* spp., *Prevotella* spp., *A. viscosus* spp., *S. equismillis* spp., *S. dygalactiae* spp., *S. sanguis* spp., *S. cohnii* spp., *B. intermedius* spp., *A. parvulum* spp., *E. saburreum* spp., *E. sulci* spp., *P. micra* spp., *S. moorei* spp., *S. agalactiae* spp., *C. minutissimus* spp., *P. propionicus* spp., *S. agalactiae* spp., *S. dysgalactiae* spp., *S. simulans* spp., *S. xylosus* spp., Tinea pedis infection causing fungi, *S. salivarius* spp. Other than K12 or M18, *L. lactis* spp., *S. epidermidis* spp., *S. constellatus* spp. or any combination of any two or more thereof.

[00251] In various embodiments, the oral and/or dental microorganism is selected from *S. intermedius* spp., *M. catarrhalis* spp., *H. influenzae* spp., *S. pyogenes* spp., *S. mutans* spp., *S. pneumoniae* spp., *F. nucleatum* spp., *A. actinomycetemcomitans* spp., *P. intermedia* spp., *Prevotella* spp., *A. viscosus* spp., *S. sobrinus* spp., *B. intermedius* spp., *A. parvulum* spp., *E. saburreum* spp., *E. sulci* spp., *P. micra* spp., *S. moorei* spp., *S. agalactiae* spp., or any combination of any two or more thereof.

[00252] In various embodiments, the skin microorganism is selected from *S. aureus* spp., *S. saprophyticus* spp., *S. pyogenes* spp., *C. acnes* spp., *C. albicans* spp. *S. cohnii* spp., *C. minutissimus* spp., *P. propionicus* spp., *S. agalactiae* spp., *S. dysgalactiae* spp., *S. simulans* spp., *S. xylosus* spp., Tinea pedis infection causing fungi, *S. epidermidis* spp., or any combination of any two or more thereof.

[00253] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. aureus* spp., *S. saprophyticus* spp., *M. catarrhalis* spp., *H. influenzae* spp., *S. pyogenes* spp., *P. aeruginosa* spp., *S. mutans* spp., *S. pneumoniae* spp., *S. salivarius* spp. Other than K12 or M18, *L. lactis* spp., *S. epidermidis* spp., *S. constellatus* spp., or any combination of any two or more thereof.

[00254] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. aureus* ATCC 6538, *S. saprophyticus* ATCC 15305, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *H. influenzae* TW5, *S. pyogenes* M76, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *P. aeruginosa* I2, *P. aeruginosa* ATCC 27853, *S. mutans* OMZ175, *S. mutans* FW75, *S. pneumoniae* D39, *S. pneumoniae* RX1, *S. pneumoniae* PK8, *S. equismillis* Bris 2, *S. dygalactiae* T277, *C. acnes* ATC 6919, *S. sanguis* K11, *S. sobrinus* OMZ176, *S. cohnii*, *S. simulans*, *L. lactis* T-21, *S. epidermidis* 11, *S. epidermidis* E30, *S. constellatus* T-29, *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, or any combination of any two or more thereof.

[00255] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. saprophyticus* ATCC 15305, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *H. influenzae* TW5, *S. pyogenes* M76, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *P. aeruginosa* I2, *S. mutans* OMZ175, *S. pneumoniae* D39, *L. lactis* T-21, *S. epidermidis* 11, *S. constellatus* T-29, *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, or any combination of any two or more thereof.

[00256] *S. salivarius* strains having anti-viral activity, particularly anti-SARS-CoV2 (Covid-19) activity, are described in PCT/NZ2021/050054 (Blis Technologies Ltd). Also described are prophylactic and therapeutic compositions and methods for treating respiratory viruses. The data shows that *S. salivarius* K12, M18 have varying anti-viral activity against SARS-CoV2, influenza A, influenza B, and respiratory syncytial virus (RSV). Accordingly, the microorganism to be inhibited herein also includes respiratory viruses such as SARS-CoV2, Influenza A, Influenza B, and Respiratory Syncytial Virus (RSV).

[00257] In various embodiments, the skin, oral, dental, mucosal, and/or ENT microorganism is a *Streptococcus* or *Staphylococcus* bacteria. In various embodiments, the *Streptococcus or Staphylococcus* bacteria is selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. pyogene*s spp., and *S. pneumoniae* spp., *S. constellatus* spp., and *S. salivarius* spp. In various embodiments, the *Staphylococcus* bacteria is selected from *S. aureus* A222, *S. saprophyticus* ATCC15305, and the *Streptococcus* bacteria is selected from *S. mutans* OMZ175, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes*

M66, *S. pyogenes* M74, *S. pneumoniae* D39, *S. salivarius* spp., e.g. *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, and *S. constellatus* T-29.

[00258] In various embodiments, the *Streptococcus* or *Staphylococcus* bacteria is selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. salivarius* spp., *S. constellatus* spp., *S. pyogenes* spp., and *S. pneumoniae* spp., *S. equisimilis* spp., *S. dysgalactiae* spp., and the *S. salivarius* is K12. In various embodiments, *Staphylococcus* bacteria is selected from *S. aureus* A222, *S. saprophyticus* ATCC15305, and the *Streptococcus* bacteria is selected from *S. mutans* OMZ175, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *S. pneumoniae* D39, *S. equisimilis* Bris 2, *S. dysgalactiae* T277, *S. salivarius* spp., e.g. *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, and *S. constellatus* T-29; and the *S. salivarius* strain is K12.

[00259] In various embodiments, the skin, oral, dental, mucosal and/or ENTR microorganism is selected from *S. aureus* spp., *S. saprophyticus* spp., *L. lactis* spp., *S. epidermidis* spp., *S. salivarius* spp., *M. catarrhalis* spp., *S. mutans* spp., *H. influenzae* spp., *S. pneumoniae* spp., *S. pyogenes* spp., *L. lactis* T-21, *S. epidermidis* 11, *S. epidermidis* E30, and *S. constellatus* T-29; the *S. salivarius* strain is K12; and the supplemental saccharide is galactose.

[00260] In various embodiments, the skin, oral, dental, mucosal and/or ENTR microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. saprophyticus* ATCC 15305, *L. lactis* T-21, *S. epidermidis* 11, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *S. mutans* OMZ175, *H. influenzae*, *S. pneumoniae* D39, *S. pyogenes* 71-698, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *L. lactis* T-21, *S. epidermidis* 11, *S. epidermidis* E30, *S. constellatus* T-29; the *S. salivarius* strain is K12; and the supplemental saccharide is galactose.

[00261] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. aureus* spp., *S. saprophyticus* spp., *L. lactis* spp., and *S. epidermidis* spp.; the *S. salivarius* strain is K12; and the supplemental saccharide is raffinose.

[00262] In various embodiments, the skin, oral, dental , mucosal and/or ENTR microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. saprophyticus* ATCC 15305, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *L. lactis* T-21, *S. epidermidis* 11, *S. salivarius* 6, *S. salivarius* 193, and *S. salivarius* 20P3; the *S. salivarius* strain is K12; and the supplemental saccharide is raffinose.

[00263] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. constellatus* spp., *S. pyogenes* spp., *S. pneumoniae* spp., *S. salivarius* spp., *S. mutans* spp., *S. saprophyticus* spp., *S. aureus* spp., *M. catarrhalis* spp., *L. lactis* spp., *H. influenzae* spp., and *P. aerug*inosa spp.; the *S. salivarius* strain is M18; and the supplemental saccharide is raffinose.

[00264] In various embodiments, the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from, *S. pyogenes* M76, *S. pneumoniae* D39, *S. mutans* OMZ175, *S. saprophyticus* ATCC 15305, *S. aureus* A222, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *L. lactis* T-21, *H. influenzae* TW5, *P. aeruginosa* I2, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *S. constellatus* T-29, *S. salivarius* 6, *S. salivarius* 193, and *S. salivarius* 20P3; the *S. salivarius* strain is M18; and the supplemental saccharide is raffinose.

[00265] In various embodiments, the bacteria are selected from *S. pyogenes* spp., and *S. pneumoniae* spp.; and the *S. salivarius* strain is M18. In various embodiments, the bacteria is selected from *S. pyogenes* 71-698, and *S. pneumoniae* D39; and the *S. salivarius* strain is M18.

[00266] In various embodiments the microorganism is a virus selected from SARS-CoV2, Influenza A, Influenza B, and RSV.

Prevention or treatment of diseases or inhibition of microorganisms

[00267] In one aspect, the invention provides a method of treating or preventing a disease or disorder comprising administering to subject in need thereof a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide; wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof; and wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00268] In one aspect, the invention relates to use of *S. salivarius* and a supplemental saccharide in the manufacture of a medicament for the treatment or prevention of a disease or disorder; wherein the *S. salivarius* is *Streptococcus salivarius* M18, *S. salivarius* K12, or a combination thereof; and wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00269] In one aspect, the invention provides a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide for use in the treatment or prevention of a disease or disorder; wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof; and wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00270] In one aspect, the invention provides a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide; wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof; and wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00271] In various embodiments, the composition or therapeutic formulation improves the inhibitory profile, and/or improves the mucoid properties of the *S. salivarius*.

[00272] In various embodiments, the disease or disorder is caused by a skin, oral, dental, mucosal, or ENTR pathogen.

[00273] In various embodiments, the disease or disorder is otitis media, sore throat, tooth decay, acute pharyngitis, tonsillitis, pneumonia, COPD, periodontal disease, gingivitis, halitosis, dental caries, sepsis, meningitis, vaginitis, body odour, acne, actinomycosis, psoriasis, erythrasma, cellulitis, impetigo, atopic dermatitis, bacteraemia, soft tissue infections, erythema, nosocomial, erythema, SARS-CoV2, Influenza A, Influenza B, and RSV, candidiasis (oral thrush), athlete's foot. Or any combination of any two or more thereof.

[00274] In various embodiments, the disease or disorder is caused by pathogenic bacteria. In various embodiments, the disease or disorder is caused by pathogenic *Streptococcus* bacteria.

[00275] In various embodiments, the disease or disorder is otitis media, sore throat, tooth decay, acute pharyngitis, tonsillitis, pneumonia, COPD, periodontal disease, gingivitis, halitosis, dental caries, sepsis, meningitis, vaginitis, body odour, acne, actinomycosis, psoriasis, erythrasma, cellulitis, impetigo, atopic dermatitis, bacteraemia,

soft tissue infections, erythema, nosocomial, erythema, or any combination of any two or more thereof.

[00276] In various embodiments, the disease or disorder is caused by a pathogenic virus.

[00277] In various embodiments, the disease or disorder is SARS-CoV2, Influenza A, Influenza B, or RSV.

[00278] In various embodiments, the disease or disorder is caused by a pathogenic fungus (e.g. yeast or skin mycoses).

[00279] In various embodiments, the disease or disorder is candidiasis (oral thrush), athlete's foot (Tinea pedis), or other Tinea infections.

[00280] In various embodiments, the subject is a mammal, including humans, dogs, cats, horses, sheep, cows and other domestic and farm animals. In various embodiments, the subject is a non-human subject. In various embodiments, the subject is a human. In various embodiments, the subject is an infant, child or adult.

[00281] In one aspect the invention also relates to a method of inhibiting a microorganism sensitive to Blis-producing *S. salivarius*, comprising administering to subject in need thereof a composition of the invention, or a therapeutic formulation of the invention. The microorganism to be inhibited may be a non-pathogenic microorganism for example *S. salivarius* spp., *S. epidermidis* spp., *L. lactis* spp. or *S. constellatus* spp. To facilitate adhesion and colonisation by *S. salivarius* used in the invention, it may be desirable to reduce populations of such other non-pathogenic microorganisms.

Raw ingredient composition

[00282] Lyoprotectants and cryoprotectants are commonly used in the manufacture of products containing BLIS-producing strains (including *S. salivarius* containing products) to protect and maintain cell viability. Lyoprotectants protect during drying, while cryoprotectants protect during freezing. The same composition can have both functions, and unless otherwise specified, the terms are used interchangeably herein. Suitable lyoprotectants or cryoprotectants will be known to a person skilled in the art.

[00283] In various embodiments, the lyoprotectant may be selected from sodium caseinate, peptone, skim milk powder, whey protein, trehalose, glycerol, betaine, sucrose,

galactose, glucose, lactose, lactitol, mannitol, maltodextrin, sodium citrate, and combinations thereof.

[00284] In various embodiments, the lyoprotectant may be a mixture of trehalose, lactitol, and maltodextrin.

[00285] In various embodiments, the composition is dairy-free. In various embodiments, the composition does not comprise any dairy-derived ingredients. In various embodiments, the lyoprotectant may be a mixture of sucrose, sodium citrate, and maltodextrin or trehalose.

[00286] In various embodiments, the composition is a powder, for example a powder which has been prepared by admixing a powder of freeze-dried *S. salivarius* with a powder of the supplemental saccharide, or by co-freeze-drying *S. salivarius* with supplemental saccharide. The inventors have found that the supplemental saccharides investigated herein do not affect the stability of freeze-dried raw ingredient powders of *S. salivarius* (data unreported).

[00287] A skilled worker would appreciate the composition may comprise other excipients including a diluent or a flow aid.

Use of raw ingredient in therapeutic formulation e.g. lozenge etc.

[00288] The composition may be formulated into therapeutic formulations for administration by various methods. A "therapeutic formulation" is a composition appropriate for use in prophylactic or therapeutic treatment of an individual in need of same. In general, therapeutic formulations are composed of a *S. salivarius* strain and supplemental saccharide discussed above and a pharmaceutically acceptable carrier, diluent and/or excipient.

[00289] In one aspect, the composition of the invention is formulated into a therapeutic formulation. In various embodiments, the composition or therapeutic formulation is formulated for oral, dental, nasal, ENTR, or topical administration.

[00290] In various embodiments, the therapeutic formulation is a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, aerosol, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel, spray, deodorant, serum, lotion, balm, moisturiser, pessary or suppository.Slow or sustained release products which maintain the

level of supplemental saccharide in the oral cavity or ENTR are preferred in some embodiments.

[00291] Slow or sustained release products which maintain the level of supplemental saccharide in the oral cavity or ENTR are preferred in some embodiments. Such slow- or sustained release products are known in the art and include multilayer tablets, slow or fast dissolving melts, films, chewing gum, gels, mucoadhesive or buccal adhesive delivery systems.

[00292] An "acceptable carrier, diluent and/or excipient" means a vehicle for delivery of a *S. salivarius* strain or extract, to a surface or a host, in which the vehicle is compatible with bacterial cell viability, or activity of the extract. Acceptable carriers, diluents and excipients suitable for use in the administration of viable streptococcal strains, particularly *S. salivarius* strains and extracts are well known to those skilled in the art (see, for example, Remington's Pharmaceutical Sciences, 22nd ed., Gennaro, ed., 2013, Mack Publishing Co., Easton, Pa.), incorporated herein by reference. Suitable carriers are generally inert and can be either solid or liquid.

[00293] In various embodiments, the carrier is a pharmaceutically acceptable carrier. Such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. A variety of pharmaceutically acceptable carriers suitable for administration of viable or lyophilized bacteria are well known in the art (See for example Remington's supra.; and the pharmaceutical composition LACTINEXä (Hynson, Westcott and Dunning, Baltimore, Md. USA), a commercially available formulation for oral administration of viable lactobacilli). Suitable solid carriers known in the art include, for example, magnesium carbonate; magnesium stearate; celluloses; talc; sugars such as maltose, fructose, sucrose, mannitol, lactose, isomalt, maltodextrin, starches; flours; oligosaccharides and skim milk, and similar edible powders, but are not limited thereto.

[00294] Typical diluents, by way of example are: starches; lactose; mannitol; kaolin; calcium phosphate or sulphate; inorganic salts such as sodium chloride; and powdered sugars or celluloses.

[00295] The therapeutic formulation may also include excipients such as resins; fillers; binders; lubricants; solvents; glidants; disintegrants; preservatives; buffers; flavourings; colourings; sweeteners; and fragrances as appropriate.

[00296] Typical binders include starch; gelatin; sugars such as lactose, fructose, and glucose; and the like. Natural and synthetic gums are also convenient, including acacia;

alginates; locust bean gum; methylcellulose; polyvinylpyrrolidine; tragacanth gum; Xanthan gum: and the like. Polyethylene glycol; ethyl cellulose; and waxes can also serve as binders.

[00297] Lubricants to prevent sticking to a die during manufacture include slippery solids such as talc, silica, magnesium and calcium stearate, polyethylene glycol, stearic acid and hydrogenated vegetable oils.

[00298] Disintegrators are substances which swell when wetted to break up the composition and release the streptococci or extract. The disintegrators include starches; clays; celluloses; algins and gums; more particularly corn and potato starches; methylcellulose; agar; bentonite; wood cellulose; cation exchange resins; alginic acid; guar gum; citrus pulp; carboxymethylcellulose; powdered sponge; and sodium lauryl sulfate.

[00299] For delivery to the respiratory tract, the composition can also be in a form for administration by inhalation. The inhaled product is typically in powdered or micronized powder form, or liquid form. The product can conveniently be administered using an inhaler, nebuliser, atomiser, or any other recognised device for delivery to the respiratory tract. Carriers for inhalable products are well known in the art and include lactose, erythritol, sorbitol, and cyclodextrin.

[00300] The therapeutic formulation can additionally contain nutrients to maintain the viability and enhance the efficacy of the bacterium in the formulation. Further ingredients useful in a composition are agents that selectively enhance growth of desirable bacteria over non desirable organisms.

[00301] In various embodiments, the therapeutic formulation comprises a buffering agent (phosphate buffers, citric acid), calcium carbonate, multivitamin, mineral (e.g. Zinc, Vitamin C and D), antioxidant (berries e.g. blackcurrant, quercetin, liquorice), fluoride, xylitol, yeast extract (*Saccharomyces cerevisiae* or *Saccharomyces boulardii*), lysate, extract (*Bifidobacterium, Lactobacillus*), and/or yogurt culture.

[00302] In various embodiments, the therapeutic formulation further comprises other potentiating agents to promote the production or activity of a composition. In various embodiments, the potentiating agents are selected from carbohydrates, for example, oligosaccharides such as Nutriose® FB (Roquette Freres, Lestrem, France), maltodextrose, and lactulose; prebiotic agents; chemicals such as reducing agents, for example cysteine and mercaptoethanol; and metal ions such as magnesium.

[00303] Therapeutic formulations can also be formulated to contain flavouring agents, colouring agents, sweeteners (xylitol, maltodextrin, monk fruit extracts, stevia, aspartame), taste-masking agents (Smoothenol®), fragrances, or other compounds which increase the appeal of the product to a patient and/or enhance patient compliance without compromising the effectiveness of the product. Methods for preparation of therapeutic formulations for inhalable administration are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 22nd ed., supra, incorporated herein by reference).

[00304] A topical therapeutic formulation may comprise other additives conventionally used in a topical composition, such as a moisturiser. Art skilled readers will further appreciate that additives need to be compatible with probiotic viability and efficacy. Such additives may provide or improve a therapeutic, cosmetic, stability, and/or appearance property of the therapeutic formulation. Examples of suitable additives include, but are not limited to, a carrier (e.g. vegetable oils, triglycerides, glycerol, propylene glycol, water, saline), a surfactant, a dispersant, an emulsifier, an inhibitory activity enhancer, a buffering agent, an antibacterial agent, a prebiotic, a fragrance, an antioxidant, a colourant, a skin protective agent, an anti-aging agent (hyaluronic acid, ceramides, olive squalene), an antimicrobial, an aluminium salt, a mineral pigment, an odour absorbant or neutraliser, or a sunscreen agent. Such additives may be included in the therapeutic formulation of the invention in amounts typical for topical formulations. A variety of pharmaceutically acceptable additives suitable for topical application of viable or lyophilized bacteria are well known in the art.

[00305] It may be advantageous to formulate the composition into a slow or sustained release composition or therapeutic formulation. In various embodiments, the composition is formulated in a slow-release composition. In various embodiments, the composition is formulated in a two-part composition that provides immediate release of the *S. salivarius* and the supplemental saccharide, and a slow release of further supplemental saccharide.

Methods of administration

[00306] The reader will appreciate that the compositions and formulations of the invention may be administered according to a wide range of protocols to inhibit microbial populations for both therapeutic and non-therapeutic purposes. Any protocols known in the art for administration of *S. salivarius* K12 and M18 may be used.

[00307] In various embodiments, the therapeutic formulation is administered orally once, twice, three, four times, or up to twelve times daily. In various embodiments, the

therapeutic formulation is administered orally via a lozenge, powder, melt, mouthwash, or toothpaste. For the treatment of halitosis, it is recommended to pre-treat with a mouthwash such as a chlorhexidine mouthwash or mechanical cleaning such as with a toothbrush.

[00308] In various embodiments, the therapeutic formulation is administered topically, as often as required, usually once or twice daily. In various embodiments, the composition is administered topically via a cream, serum, deodorant, spray, or moisturizer. It may be recommended to pre-treat the skin with water, soap, or a cleansing formulation prior to administration.

[00309] In various embodiments, the therapeutic formulation is administered rectally or vaginally, as required, usually once or twice daily via pessary or suppository.

[00310] In various embodiments, the therapeutic formulation is administered via a pulmonary route e.g. by nebuliser or inhaler, as often as required, usually once or twice daily.

[00311] The therapeutic formulation is useful for improving the oral health of a subject. For example, by preventing or treating any of the conditions identified in WO2001027143, WO2002070719, and WO2005007178 (supra), and all incorporated herein by reference in their entireties. *S. salivarius* M18 is also known to help reduce dental plaque, support oral health and oral flora, reduce dental caries, prevent dental caries, treat and prevent gingivitis, and treat and prevent periodontitis (Burton, J.P., et al., 2013 *J. Med. Microbiol.* 62, 875–884; Burton, J.P., et al., 2013, *PLoS ONE 8.*; Di Pierro, et al. 2015. *Clin Cosmet Investig Dent.*; 7:107-13; L Scariya, D.V, N., M Varghese, 2015. *Int. J. Pharma Bio Sci.* 6, 242–250).

Method of manufacturing ingredient

[00312] In one aspect, the invention provides a method of manufacturing a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising:

(a) combining *S. salivarius* with supplemental saccharide, and

(b) mixing to produce a homogeneous blend;

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00313] In various embodiments, the *Streptococcus salivarius* is in the form of a powder. In various embodiments, the saccharide is in the form of a powder, for example a freeze-dried powder. In various embodiments the powder is a raw ingredient powder comprising the *S. salivarius* and lyoprotectant as discussed above.

[00314] In various embodiments, the mixing occurs in a blender. In various embodiments, the product is then packaged.

[00315] In various embodiments, excipients are added.

[00316] In various embodiments, the inhibitory profile of the S. *salivarius* in the composition is improved.

[00317] In various embodiments, the composition is for use in the treatment or prevention of a disease or disorder, including the diseases and disorders referenced above.

[00318] In various embodiments, the composition is for use in inhibiting a microbial population sensitive to a Blis-producing *S. salivarius*.

[00319] In one aspect, the invention relates to the use of a composition manufactured by the method of the invention for the treatment or prevention of a disease or disorder.

[00320] In one aspect, the invention relates to the use of a composition manufactured by the method of the invention for inhibiting a microbial population sensitive to a Blis-producing *S. salivarius*.

Method of manufacturing formulations

[00321] In one aspect, the invention provides a method of manufacturing a therapeutic formulation comprising a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) mixing excipients,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) blending to provide the therapeutic formulation

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00322] The method may include further processing steps to provide the therapeutic formulation. A skilled worker will appreciate the required excipients depending on the administration route and blending methods such as homogenisation.

[00323] In various embodiments, the invention provides a method of manufacturing an oral lozenge comprising a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) mixing a carrier, tableting aids (e.g. binder, lubricant), and flavouring agent,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) blending the mixture, and

d) lozenging the mixture to provide the lozenge

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00324] The inventors have determined that the supplemental saccharides investigated herein do not affect the stability of *S. salivarius* in the lozenges (data unreported).

[00325] In various embodiments, the invention provides a method of manufacturing an oral powder comprising a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) mixing a carrier, tableting aids (e.g. binder, lubricant), and flavouring agent,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) blending the mixture to provide the oral powder

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00326] In various embodiments, the invention provides is a method of manufacturing a topical composition comprising a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) mixing an oil vehicle and dispersing agent,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) homogenising the mixture to provide the topical composition,

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00327] In various embodiments, the invention provides a method of manufacturing a pessary or suppository comprising a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) mixing a solid lipid with other excipients,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) homogenising the mixture to provide the pessary or suppository,

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00328] In various embodiments, the invention provides a method of manufacturing a formulation for pulmonary administration comprising a composition comprising *S. salivarius* and an effective amount of a supplemental saccharide, the method comprising

a) optionally mixing a dry powder carrier with other excipients,

b) adding a composition comprising *S. salivarius* and supplemental saccharide of the invention, and

c) mixing to provide the formulation for pulmonary administration,

wherein the *S. salivarius* is *S. salivarius* M18, *S. salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

[00329] The following non-limiting examples are provided to illustrate the present invention and in no way limit the scope thereof.

EXAMPLES

Materials

[00330] The following culture media and saccharide were all supplied by Fort Richard Laboratories, New Zealand: CABK12 agar plates; Columbia Blood Agar Base (CAB) bottles; Colombia agar base with 0.5% *(w/v)* $CaCO_3$ (CABCa). Columbia agar Base supplemented with 0.1% $CaCO_3$ (and 5% v/v sheep blood (sBaCa); Haemophilus agar plates Mitis Salivarius Agar; D-galactose - BD Difco; Todd Hewitt Broth (THB) - BD Difco (used as per manufacturers instruction); M17 Broth – BD Difco (used as per manufacturers instruction, but no saccharide added).

[00331] The following materials were all supplied by Lab Supply Ltd, New Zealand: Calcium carbonate ($CaCO_3$) - PanReac Applichem; D-(+) Glucose – monohydrate – Applichem; Ethanol (96% AR Grade) – diluted with water to produce 70% solution prior to use. D (+)-Glucose monohydrate – Applichem, Chloroform (Emsure, ACS, ISO, Reag. Ph Eur) – Merck. Water for molecular biology (Nuclease free) – AppliChem, 10X Tris-Acetate-EDTA (TAE) buffer – Applichem. Hydrochloric acid (HCl).

[00332] D-(+) Raffinose pentahydrate; D-(-) Fructose (both from Sigma, New Zealand).

[00333] Chloroform (Emsure, ACS, ISO, Reag. Ph Eur) – Supelco (Sigma Aldrich, New Zealand).

[00334] The following materials were all supplied by Thermo Fisher Scientific, New Zealand: PureLink RNA Mini Kit; TRIzol reagent; Phasemaker tubes; RNaseZap; TURBO DNA-free kit; SuperScript IV VILO; PowerTrack SYBR Green master Mix; 10,000X SYBR safe DNA gel stain; MicroAmp Optical 384-Well reaction plate; MicroAmp Optical adhesive film and Phosphate Buffered Saline (PBS) - Oxoid

- Primers for qPCR:
  - K12recA_fw TGAGTCAAGCCATGCGTAAG
  - K12recA_rv CAGGGGTCGTTTCTGGATTA
  - K12gyrA_fw (5'-CCGTTACATGTTGGGTTGACG) –
  - K12gyrA_rv (5'-ATCACGAAGCATTTCCAAGG)
  - 18K12salA_fw TGATTGCCATGAAAAACTCAA
  - M18K12salA_rv TTGCAAACCAACCTGTACCTC
  - K12SalQ_fw ACACTCGATCTCGAGGCACT
  - K12salQ_rv GAATGTGGCATAACCAACTA
  - K12salB_fw CTCGTTGAGATTGAAGCAATGA
  - K12salB_rv TTCGTGTGAAATGGTTTGGA
  - K12ureC_fw TTTCCAAGGTCGTACCGTTC
  - K12ureC_rv TGGATCCAAGTGGTGACAAA
- Primers for PCR:
  - SalBF GTGAATTCTCTTCAAGAATTGACTCTT
  - SalBR AAAATATTCATACCGCTCTTCC

[00335] Phosphate Buffered Saline (PBS) - Dulbecco A - Oxoid (supplied by Thermo Fisher Scientific, New Zealand and used as per manufacturer's instructions). AnaeroGen sachets – Oxoid - supplied by Thermo Fisher Scientific, New Zealand.

[00336] Promega GoTaq G2 Hot start Green Master mix supplied by Invitro Technologies, New Zealand.

[00337] Maestrogen AccuRuler 1kb DNA RTU ladder supplied by Mediray, NZ.

[00338] Similac 360 Total Care infant formula (Abbott Global, US) was purchased via www.Amazon.com. Whole milk powder (Anchor Blue™ Milk powder, Anchor, NZ.)

[00339] *S. salivarius* strains K12 and M18 – available from Deutsche Sammlung von Mikroorganism und Zellkulturen (DSM), sourced from Blis Technologies Ltd, New Zealand; K12 raw ingredient product (K12 powder with trehalose/lactitol/maltodextrin), M18 raw ingredient product (M18 powder with trehalose/lactitol/maltodextrin); K12 and M18 Dairy free raw ingredient products (powder with sucrose, sodium citrate, and maltodextrin lyoprotectant); K12 containing commercial powder formulation, Daily Defence Junior (DDJ) (Composition: *S. salivarius* K12, Isomalt, Maltodextrin, Vanilla flavour) K12 containing commercial lozenge formulation (Throat Guard Pro) (composition: *S. salivarius* K12, isomalt, tableting aids, natural flavour) - all from Blis Technologies Ltd, New Zealand); *S. saprophyticus* ATCC15305, *S. mutans* NCTC 10449 (ATCC 25175); *S. mutans* UA159 (ATCC700610); *S. cohnii* (ATCC29974); *S. simulans* (ATCC 27848)*; C. acnes 6919; F. nucleatum* ATCC 25586; *P. gingivalis* ATCC 33277; *P. gingivalis* ATCC 53978; *P. intermedia* ATCC 25611; *S. salivarius* (ATCC 13419, ATCC25923 and ATCC 7073); *S. sobrinus* ATCC 27351 *S. agalactiae* ATCC 12386, *A. viscosus* ATCC 15987 *C. auris* ATCC 51966– all available from American Type Culture Collection (ATCC); *M. catarrhalis* TW1 and TW2; *L. lactis* T-21; *S. pyogenes* M76; *P. aeruginosa* I2; *H. influenzae* TW5; *S. aureus* A222; *S. mutans* OMZ175; *S. pneumoniae* D39; *S. pyogenes* 71-698; *S. constellatus* T-29; *S. pyogenes* FF22; *S. pyogenes* W-1; *S. pyogenes* M17; *S. pyogenes* M57; *S. pyogenes* EMM92; *S. pyogenes* M66; *S. pyogenes* M74; *S. pyogenes* H13; *S. pyogenes* K26*; S. pyogenes* WS02*, S. dysgalactiae* Bris 2; *S. dysgalactiae* T277; *S. pneumoniae* RX1; *S. pneumoniae* PK8; *S. mutans* D10; *S. mutans* FW75; *A. viscosus* T14; *S. sanguis* K11; *S. aureus* 20; *S. aureus* 19; *S. aureus* 14; *F. nucleatum* FH2; *F. nucleatum* FH3; *S. salivarius CN3410; 34A; K14; H29; S. pyogenes K7; S. dysgalactiae T-148; S. aureus 19* – all available from Blis Technologies Ltd on request. *S. sobrinus* OMZ176 (CCUG21020) – available from Culture Collection University of Gothenburg (CCUG); *S. pyogenes* 71-679 – standard gifted from Lewis Wannamaker.

Method 1 – Preparing solid culture media

[00340] Calcium carbonate ($CaCO_3$) were added to all solid culture media (CAB) during preparation as a buffering agent. $CaCO_3$ was present in all prepared culture media at a 0.5% (*w/v*) concentration (CABCa)..

[00341] Premade bottles of Columbia agar base (CAB) agar were ordered from Fort Richard Laboratories, New Zealand in 180mL volumes. Additional ingredients were added to these 180mL bottles. Carbohydrates were added to separate bottles of CAB agar at 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% (*w/v*) concentrations. The following equation was

used to determine the amount of carbohydrate (and $CaCO_3$) to add to achieve a desired concentration of that ingredient:

[00342] $$Volume\ of\ Dissolvent\ (mL) \times \left(\frac{\left(desired\ contration\ (\%)\left(\frac{w}{v}\right)\right)}{100}\right) =$$

$$amount\ of\ solute\ to\ add\ (g)$$

[00343] For example, to obtain a CAB agar base containing $CaCO_3$ and galactose at a 0.5% (*w*/v) concentration using one of the premade Fort Richard agar bottles the following equation would be used:

[00344] $$180\ (mL) \times \left(\frac{0.5\ (\%)\left(\frac{w}{v}\right)}{100}\right) = 0.9\ (g)$$

[00345] A precise method was used to ensure sterility of the culture media and that accurate amounts of any additive ingredients were correctly integrated into the culture medium. Ingredients were removed from their containers using clean spoons/spatulas and were weighed in clean weigh boats on a balance which measured to the milligram. Once the ingredients had been weighed out, they were tipped into clean 30ml containers, labelled, and sealed. Premade CAB agar is solid when it arrives from Fort Richard, therefore a crater approximately 3cm wide and 3cm deep must be cut into the agar using a sterile scalpel to create a space for the ingredients to be poured into. Following this the ingredients were poured into the crater and 1ml of sterile distilled water was dispensed on top to partially soak the ingredients into the agar. Agar that was cut away to create the cavity was then replaced within the crater and a magnetic stirrer is placed into the bottle. The bottle was then autoclaved at 110°C for 10 minutes. The bottle was stored in a water bath set to 50°C. Sterile petri dishes were labelled with the appropriate information (date, contents of the plate, and added ingredients) and placed into the Class II Biological Safety Hood with their lids off. Prior to pouring the agar into the petri dishes, the contents were stirred thoroughly for 1-2 minutes using the magnetic stirrer. A Bunsen flame was lit inside the Biological Safety Cabinet as a further sterility measure and the contents of the bottle was poured out into the petri dishes. If any bubbles appeared while pouring, they were popped using the Bunsen flame. The agar was left for 5-10 minutes to set. One plate was labelled “negative control” and placed into the incubator overnight to ensure no contamination occurred during agar preparation.

Method 2 – Deferred antagonism assay

[00346] 1-2 producer strain colonies were transferred from a stock plate into 900µL of suspension solution using a fresh cotton swab. Following this, suspensions were vortexed, and a sample was inoculated onto solid culture media using a fresh cotton swab. The inoculation was a streak that runs diametrically across the agar plate in a 1.5cm wide strip. The plate was then incubated. Following incubation all visible colonies were removed by swabbing them from the agar surface. The plates were then treated with chloroform by dispensing 2ml of chloroform onto 4cm × 4cm piece of cloth and sealing the inverted agar plate on top for 30 minutes. The plates were then air dried for 30 minutes following chloroforming. 1-2 indicator strain colonies were then transferred from a stock plate into complementary suspension solution. The suspensions were then vortexed, and a sample was inoculated onto the same agar surface. These samples were streaked in a perpendicular direction to the initial producer streak.

[00347] Each indicator streak was prepared with fresh a suspension containing cells from a corresponding stock plate containing the indicator strain of interest. Indicator strains suspension samples were transferred using a fresh cotton swab. Results were measured by qualitatively reviewing the subsequent (if any) sizes of the zones of inhibition (ZOI) of indicator strain colony growth. A positive result is when there is a distinctive reduction or complete inhibition of indicator colony growth on the culture medium. A negative result is when there is no suggestion of a reduction in the indicator strain colony density.

Method 3 - Dose response assay

[00348] CABCa agar plates containing various concentrations of saccharide were prepared according to the methods described in Method 1. Producer strains were transferred from stock agar plates into 900µL THB using a fresh cotton swab. A plastic spread plater was cut to approximately 1.2cm width and then dipped into ethanol and left to air dry to ensure sterility. A 100µL sample of the producer strain suspension was then dispensed onto the agar surface in a straight line running diametrically across the agar surface in (approximately) 20µL spots. The spots were spaced approximately 1.5cm apart from each other. The plastic spreader was then used to evenly distribute the spots across the agar surface in a 1.5cm wide strip. This step was taken to ensure an even producer streak was inoculated onto the agar surface and provided an approximate measure of the initial inoculum concentration. Separate plastic spreaders were used for samples containing different producer strains. The samples were left to soak into the agar surface

until there was no visible liquid remaining on the agar surface (approximately 20 minutes). Following producer streak preparations, the deferred antagonism assay protocol was followed as per Method 2. Results were recorded by measuring the size of the zone of inhibition (ZOI) in mm.

Example 1: Antagonism of ENTR microorganisms by K12

[00349] This example shows inducement of antagonism of ENTR microorganisms by K12 and supplemental saccharide.

[00350] BLIS K12™ cultured onto CABCa agar plates containing 0.1%, 1.0%, 1.5%, and 2.0% (*w/v*) concentrations of galactose and raffinose were studied for their antimicrobial activity by a deferred antagonism assay according to the protocols outlined in Method 3. The results presented in **Figure 1** and **Figure 2** illustrate the change in ear, nose, and throat (ENT) microorganism indicator strain zone of inhibition sizes. When K12 was cultured in the presence of various concentrations of galactose the ZOI sizes for several of the ENTR indicator strains increased markedly from the control condition (**Figure 1**). *M. catarrhalis* TW1 and TW2 display similar changes in the ZOI sizes across the various concentrations of galactose. The change in TW1 ZOI size peaks at 1.5% galactose(*w/v*) (12mm) then decreases at 2.0% galactose (*w/v*), whereas the change of TW2 ZOI size peaks at 2.0% galactose (*w/v*) (11mm). The change in *L. lactis* T-21 ZOI sizes trend upwards relative to the concentration of galactose present in the culture medium.

[00351] *S. pyogenes* M57 ZOI sizes increase from the control condition at 0.1%, 1.0%, and 1.5% (*w/v*) concentrations of galactose. The change in M76 ZOI size remains level (11mm) between 1.5% and 2.0% concentrations of galactose. The concentration of galactose that produces the largest ZOI change (on average) is 2.0% (*w/v*) (Average ZOI change @ 2% galactose= 9.5mm).

[00352] When K12 was cultured on CABCa incorporated with various concentrations of raffinose during a deferred antagonism assay, a change in the ZOI sizes from a control condition (no carbohydrate) was demonstrated for several ENT microorganism indicator strains (**Figure 2**). The change in *L. lactis* ZOI size from the control condition to 0.1% raffinose (*w/v*) was 15mm. This large increase was followed by an increase to 16mm at 1.0% raffinose (*w/v*) (indicating significantly greater inhibition), where the ZOI size then levels off and eventually decreases at 2.0% raffinose (*w/v*). The change in ZOI size for *H. influenzae* TW5 and *S. pyogenes* M76 also peaked at 1.5% raffinose (*w/v*) at 12mm and

13mm, respectively. TW5 ZOI size actually returned to the same as the control condition (0mm) at 2% raffinose (*w/v*). *M. catarrhalis* TW1 and TW2 ZOI sizes changed very similarly between conditions (**Figure 2**). The changes in ZOI size for each strain peak at the end of our range of study (2.0% (*w/v*) (13mm). The concentration of galactose that produced the largest ZOI size change (on average) was the 1.5% raffinose (*w/v*) condition (Average ZOI change @ 1.5% raffinose = 10.33mm).

Example 2: Antagonism of ENTR microorganisms by M18

[00353] This example shows inducement of antagonism of ENTR microorganisms by M18 and supplemental saccharide.

[00354] BLIS M18™ cultured onto CABCa agar plates containing 0.1%, 1.0%, 1.5%, and 2.0% (*w/v*) concentrations of galactose and raffinose were studied for their antimicrobial activity by conducted a deferred antagonism assay according to the protocols outlined in Method 3. The results presented in **Figure 3** and **Figure 4** illustrate the change in ear, nose, and throat (ENT) microorganism indicator strain zone of inhibition sizes.

[00355] When M18 was cultured on CABCa incorporated with various concentrations of galactose there was a subsequent change in the ZOI sizes for several ENTR microorganism indicator strains during a deferred antagonism assay (**Figure 3**). *M. catarrhalis* strains TW1 and TW2 both demonstrated increases in their respective zones of inhibition when galactose was incorporated in the culture media at 1.0% and 1.5% (*w/v*) concentrations versus the control condition (12 → 23mm and 13 → 23mm, respectively). Both *L. lactis* and *S. pyogenes* M76 zones of inhibition were the largest when galactose was present at a 1.5% (*w/v*) concentration (9mm and 14mm, respectively). These were marked differences from their control condition ZOI sizes. All susceptible ENTR indicator strains showed a reduced ZOI size when galactose was present at a 2.0% (*w/v*) concentration compared to the 1.5% (*w/v*) concentration condition. The condition which produced the largest ZOI change, on average, was the 1.5% galactose (*w/v*) condition. Average ZOI change @ 1.5% galactose = 11.5mm.

[00356] When M18 was cultured on CABCa agar incorporated with various concentrations of raffinose a very similar pattern emerged to what was observed in ZOI sizes in the galactose conditions. However, in the raffinose conditions, more ENTR indicator strains exhibited some inhibition and the ZOI sizes (on average) were greater than the corresponding ZOI sizes in the galactose conditions. *M. catarrhalis* strains TW1 and TW2 zones of inhibition displayed the largest change in size from the control

condition when raffinose was present at a 1.5% (*w/v*) concentration (31mm, respectively). Contrary to the trend observed in the galactose condition, *L. lactis* ZOI size was greatest in the 0.1% (*w/v*) raffinose condition (20mm). *P. aeruginosa* I2 ZOI size did not change from the control condition across any of the raffinose conditions, excluding at 1.5% (*w/v*) concentration where the ZOI = 10mm in width. *H. influenzae* TW5 ZOI size was greatest in the 1.5% (*w/v*) raffinose condition (26mm). Again, all susceptible ENTR indicator strain ZOI sizes exhibited marked reductions in width when raffinose was incorporated into the culture media at a 2.0% (*w/v*) concentration (**Figure 4**). The condition which produced the largest ZOI change, on average, was the 1.5% raffinose (*w/v*) condition. Average ZOI change @ 1.5% raffinose = 22.16mm.

[00357] When the same experiment was conducted with K12-/- (a strain that does not have the bacteriocin encoding megaplasmid) there were no zones of inhibition formed for any of the ENTR indicator strains.

Example 3: Activity of raffinose compared to Trimix (mixture of equal molar concentrations of the three saccharides) and individual saccharides

[00358] This example shows that the stimulatory effect of K12 and M18 was due to raffinose and not to equimolar amounts of one or all of its individual constituents, i.e. the raffinose is not being metabolised to individual constituents that are having effect. Further to this, the effect of any change in pH from the assay design could have had in influencing the change in inhibitory effects was assessed.

[00359] A dose response assay was carried out according to method 3 above, except a 1.2cm streak was used.

[00360] **Figures 5-7** highlight the enhanced efficacy of BLIS K12 or M18 + raffinose, trimix and individual saccharides against skin, dental, ENTR, pathogens.

[00361] **Conclusions:** The comparative assessment of equimolar concentration of raffinose vs trimix saccharides and individual saccharides showed that in presence of raffinose, K12 has better inhibitory activity against microorganisms associated with skin, dental, ENTR, compared to an equivalent composition of the three monomeric saccharides. The effect seems to be mediated via enhanced production of antimicrobial molecules (such as bacteriocins or non-ribosomal peptides) and not an inhibitory effect due to drop in pH.

Example 4: Activity of raffinose compared to Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides

[00362] This example shows that the stimulatory effect of K12 and M18 was due to raffinose and to equal percent weight amounts of one or all of its individual saccharides, i.e. the raffinose is not metabolised to individual saccharides that are having this effect.

[00363] A dose response assay was carried out according to method 3 above, except a 1.2cm streak was used. For the individual saccharide testing the pH of the producer streak was adjusted after the growth of producer to change the acidic conditions.

[00364] The enhanced efficacy of K12 or M18 + (% w/v) with raffinose over an equal weight percentage amount of saccharides either as a trimix or as individual saccharides was seen against representative microorganism from skin, dental, lower respiratory diseases as well as other *S. salivarius* **(Figure 8 -13)**. The activity is normalised to baseline control.

Example 5: Raffinose promotes antimicrobial (e.g. bacteriocin or non-ribosomal peptides) activity of K12 and M18 rather than acid production

[00365] The example shows that the stimulation of greater inhibition of these microorganisms was due to an increase in production of antimicrobial molecules (bacteriocins or non-ribosomal peptides) and not due to an associated decrease in pH to a more (and therefore potentially inhibitory) acidic environment.

[00366] **Method:** Bacterial test strains were assessed for their ability to grow on an equivalent agar to Columbia agar base without calcium carbonate at different pH's ranging from pH 4.5 to pH 7. The bacterial test strains were suspended in either Todd Hewitt broth or a relevant growth media for the strain, before being swabbed across the test plates at different pH's. These plates were then incubated for at 37°C with 5% CO_2, bacterial growth for each test strain was monitored at 18 and 24h.

[00367] **Results:** At 24h all the strains tested grew at pH 7 but as the pH dropped the number of strains able to grow decreased (Table 1). Only 3 out of the 9 strains grew at pH 5.25 or below. This shows that production of acid by either K12 or M18 grown on CABCa supplemented with trimix or individual saccharides could either reduce or totally inhibit the growth of the strains being tested.

Table 1.

		pH								
Indicator microorganism	strain	4.5	4.75	5	5.25	5.5	5.75	6	6.5	7
1. *S. saprophyticus*	ATCC 15305	-	LG	+	+	+	+	+	+	+
2. *S. mutans*	OMZ175	-	LG	LG	LG	+	+	+	+	+
3. *S. pneumoniae*	D39	-	-	-	-	-	-	LG	LG	+
4. *S. constellatus*	T-29	-	-	-	LG	LG	+	+	+	+
5. *S. pyogenes*	71-698	-	-	-	-	+	+	+	+	+
6. *H. influenzae*	TW5	-	-	-	-	-	-	-	-	+
7. *M. catarrhalis*	TW1	-	-	-	-	-	-	-	+	+
8. *S. aureus*	A222	+	+	+	+	+	+	+	+	+
		- = no growth, + = growth, LG = less growth								

[00368] Note that raffinose did not induce significant drop in pH (> 5.25) but results in inducing K12 to produce inhibitory activity. Observed inhibitory activity of K12 in presence of raffinose is due to antimicrobial activity, not a pH effect.

Example 6: Activity of raffinose with K12 or M18 compared to Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides.

[00369] This experiment shows that the inhibitory spectrum of K12 was found to be extended to species and strains, not typically inhibited by K12 (Table 2 and 3). K12 was induced by raffinose specifically against skin microorganisms, highlighting the potential benefit of K12 to skin indications such as impetigo, atopic dermatitis etc.

[00370] The method used was the same as Example 4.

[00371] **Results** The below Table 2 highlights which species/strains became sensitive to the inhibitory molecules (e.g. bacteriocins) from K12 that has been incubated with raffinose (Table 2). Considering for example 2.5% raffinose would contain equal percentage (0.83% each of the three saccharides). Similar results were obtained for M18.

Table 2.

	Inhibitory activity of *S. salivarius* K12 in presence of saccharides					
Indicator strains	No saccharide (Control)	Raffinose (Range: 2.5% - 10% w/v)	Trimix (Range: 2.5% - 10% w/v)	Galactose (Range: 0.83% - 3.3% w/v)	Fructose (Range: 0.83% - 3.3% w/v)	Glucose (Range: 0.83% - 3.3% w/v)
S. aureus A222	x	✓	x	✓	x	x
S. saprophyticus ATCC 15305	x	✓	x	x	x	x
S. mutans OMZ175	x	✓	✓	x	x	x
Total strains inhibited	0/3	3/3	1/3	1/3	0/3	0/3

[00372] This effect shown in Table 3 below was seen at a nominated percentage of raffinose and equivalent trimix (by percent mass) or individual saccharides

Table 3.

	Inhibitory activity of *S. salivarius* K12 in presence of saccharides					
Indicator strains	No saccharide Control	Raffinose (3.3% w/v)	Trimix (3.3% w/v)	Galactose (3.3% w/v)	Fructose (3.3% w/v)	Glucose (3.3% w/v)
S. aureus A222	x	✓	x	x	x	x
S. saprophyticus ATCC 15305	x	✓	x	x	x	x
S. mutans OMZ175	x	✓	x	x	x	x
Total strains inhibited	0/3	3/3	0/3	0/3	0/3	0/3

[00373] K12 or M18 in presence of raffinose showed enhanced potency when compared with equimolar or equal percentage weight concentrations of trimix saccharides or saccharides alone.

[00374] The inhibitory activity of raffinose is attributed to induction of K12 or M18 to produce bacteriocin rather than or in addition to acid production due to metabolism of simple saccharides (acid production in the oral cavity can cause dental caries and provide safe niche for acid tolerant bacteria such as *S. mutans*).

[00375] A selective dose related inhibitory effect was observed with raffinose suggesting a specific dose level of raffinose is required to achieve the benefits rather than a blanket prebiotic effect with common saccharides alone or in combination.

[00376] Specific observations:

- Indicator strain *S. aureus* A222 went from being insensitive to K12 to being sensitive once 1.25, 2.5, 5 and 10 % w/v raffinose was added to K12.

- Indicator strains *S. mutans* OMZ175, and *S. saprophyticus* ATCC 15305 went from being insensitive to K12 to being sensitive once 2.5, 5 and 10 % w/v raffinose was added.

- Indicator strain *S. mutans* OMZ175 went from being insensitive to M18 to being sensitive once 0.5, 1.25, 2.5, and 5% w/v raffinose was added.

- Indicator strains *S. constellatus* T-29, *S. aureus* A222, and *S. saprophyticus* ATCC 15305 went from being insensitive to M18 to being sensitive once 1.25, 2.5, 5% w/v raffinose was added.

[00377] An increase in inhibition by K12 was seen for:

- *S. constellatus* T-29 with 0.5 to 10% w/v raffinose
- *S. pyogenes* with 0.5 to 10% w/v raffinose
- S. *pneumoniae* with 0.5 to 10% w/v raffinose

[00378] An increase in inhibition by M18 was seen for:

- *S. pyogenes* with 0.5 to 10% w/v raffinose
- S. *pneumoniae* with 0.5 to 10% w/v raffinose

Example 7A: Effect of concentration of raffinose to induce inhibitory activity of K12 or M18

[00379] This example shows the effect of concentration of raffinose for enhanced inhibitory effects. Raffinose concentrations of 1.7, 2.5, 3.3, 5 and 10% were compared in Table 4 – 7 below.

[00380] Blank spaces in the table are where no result was obtained due to contamination of the test.

Table 4 – K12

Producer	Saccharide	% wt	*S. constellatus* T29	*S. pyogenes* 71-698	*S. mutans* OMZ 175	*S. pneumoniae* D39	*S. saprophyticus* ATCC 15305	*M. catarrhalis* TW1	*S. aureus* A222	Total number of strains inhibited
K12	Raffinose	1.7 %	8	10	11	5	7	0	12	6
		2.5 %	22	9	16	8	12	10	15	7
		3.3 %	--	11	16	10	12	0	16	5
		5 %	--	7	16	8	13	5	14	6
		10 %	2	3	15	10	12	6	13	6

Table 5 – K12 (pH adjusted to counter inhibitory effect due to acidic pH)

Producer	Saccharide	%wt	*S. constellatus* T29	*S. pyogenes* 71-698	*S. mutans* OMZ 175	*S. pneumoniae* D39	*S. saprophyticus* ATCC 15305	*M. catarrhalis* TW1	*S. aureus* A222	Total number of strains inhibited
K12	Raffinose	1.7%	4	4	7	10	0	0	8	5
		2.5%	18	3	13	9	8	0	15	6
		3.3%	16	--	14	7	11	0	13	5
		5%	12	1	15	13	12	0	14	6
		10%	11	3	10	10	11	0	13	6

Table 6 – M18

Producer	Saccharide	%wt	*S. constellatus* T29	*S. pyogenes* 71-698	*S. mutans* OMZ175	*S. pneumoniae* D39	*S. saprophyticus* ATCC 15305	*M. catarrhalis* TW1	*S. aureus* A222	Total number of strains inhibited
M18	Raffinose	1.7%	22	--	22	3	16	7	20	6
		2.5%	18	8	20	10	17	17	20	7
		3.3%	18	8	21	7	17	0	18	6
		5%	16	5	19	2	14	15	16	7
		10%	12	0	0	1	0	0	0	2

Table 7 – M18 (pH adjusted to counter inhibitory effect due to acidic pH)

Producer	Saccharide	%wt	*S. constellatus* T29	*S. pyogenes* 71-698	*S. mutans* OMZ175	*S. pneumoniae* D39	*S. saprophyticus* ATCC 15305	*M. catarrhalis* TW1	*S. aureus* A222	Total number of strains inhibited
M18	Raffinose	1.7%	17	10	21	12	15	0	17	6
		2.5%	16	7	19	11	15	0	18	6
		3.3%	17	7	17	13	14	0	17	6
		5%	14	5	16	12	12	0	13	6
		10%	0	0	0	11	0	0	0	1

[00381] Raffinose concentrations of 1.7, 2.5, 3.3 and 5% were all able to inhibit at least 7 of the indicators tested.

[00382] Inhibition of indicator *M. catarrhalis* was seen in Table 6 and but not in Table 7 where the agar pH was re-adjusted after growth of the producer, so this suggests that the inhibition was due to production of acid by the producers.

[00383] For most of the indicators, the largest zones of inhibition were measured on the agar plates supplemented with 2.5% raffinose.

Example 7B: Effect of concentration of galactose, combination of galactose/raffinose

[00384] Aim: to determine the effect of concentration of galactose to induce the inhibitory activity of K12 and M18.

[00385] **Method**: CABCa agar plates were prepared with or without 0.5, 1.25, 1.7, 2.5, 3.3 and 5% galactose by the addition of 0.5% (w/v) calcium carbonate to solid CAB agar in bottles and then melted by autoclaving at 110°C for 10mins. Once cooled, filter sterilised solutions of the saccharides or distilled water were added and mixed and then 20ml pipetted into petri dishes.

[00386] A deferred antagonism assay was conducted using K12 or M18 raw ingredient suspended in Todd Hewitt broth (THB). This was spread as a 1.2cm streak containing approximately 1-2x10^6 cfu down the middle of a CABCa control or CABCa galactose supplemented test plate. After 18hrs growth at 37°C with 5% CO_2, bacterial growth was removed, and the pH of the agar in the initial streak was measured and adjusted to a pH of 6.5 -7.5 using 0.5M sodium carbonate pH11 before sterilizing the agar surface using chloroform vapor. Bacterial test strains were suspended in THB before being swabbed across the plates perpendicular to the initial streak. Plates were incubated for a further 18hrs incubation at 37°C with 5% CO_2 before measuring (mm) any zones of inhibition for each test strain.

[00387] Result and Conclusion: Surprisingly, only at a concentration of 0.5% w/w of Galactose, Blis K12 and Blis M18 showed enhanced inhibitory activity (potency) and spectrum (number of pathogenic strains) activity against pathogens implicated in ENTR and skin infections (Table 8). This can be concluded that just a mere presence of Galactose may not be enough BUT a specific concentration of Galactose is required to induce the inhibitory activity.

Table 8: Effect of different concentrations of Galactose on the inhibitory activity of K12 and M18

		Size of zone inhibition normalised to control (mm)							
Producer	**Galactose (%w/w)**	***S. constellatus* T29**	***S. pyogenes* 71-698**	***S. mutans OMZ 175***	***S. pneumoniae D39***	***S. saprophyticus ATCC 15305***	***S. aureus* A222**	***S. sobrinus OMZ 176***	***Total stains inhibited***
K12	**0.5%**	14	4.5	10	8	0	12	nr	5/6
	1.25%	15	3.5	10	3	0	8	0	5/7
	1.7%	12	3.5	0	2	0	9	0	4/7
	2.5%	13	5.5	0	6	0	6	0	4/7
	3.3%	10	1.5	0	0	0	0	0	2/7
	5%	0	0	0	0	0	0	0	0/7
M18	**0.5%**	0	19.5	16	8	5	10	0	5/7
	1.25%	8	13.5	0	5	0	6	0	4/7
	1.7%	0	9.5	0	0	0	0	0	1/7
	2.5%	16	16.5	0	6	0	0	0	2/7
	3.3%	22	9.5	6	2	0	6	0	5/7
	5%	17	9.5	0	0	0	0	0	2/7

nr = no result due to contamination

Example 7C: Effect of concentration of a combination of raffinose and galactose to induce inhibitory activity of K12 or M18

[00388] This experiment aimed to determine the effect of concentration of a combination of raffinose and galactose to induce the inhibitory activity of K12 and M18.

[00389] **Method**: As above for Example 7B, except the CABCa agar plates were prepared with or without the following combinations of raffinose and galactose:

Combination No.	Percentage of Raffinose	Percentage of Galactose
1	0.5%	0.5%
2	0.5%	2.5%
3	1.25%	1.25%
4	1.25%	1.7%
5	1.25%	2.5%
6	1.7%	1.25%
7	1.7%	1.7%

8	2.5%	0.5%
9	2.5%	1.25%
10	2.5%	2.5%

[00390] **Results**: As shown in Table 9, activity against a range of pathogens was observed for various combinations of weight percentage raffinose and galactose.

Table 9: Effect of different combinations of raffinose and galactose on the inhibitory activity of K12 and M18.

			Size of zone inhibition normalised to control (mm)							
Producer	Raffinose (w/w)	Galactose (w/w)	*S. constellatus* T29	*S. pyogenes* 71-698	*S. mutans OMZ175*	*S. pneumoniae D39*	*S. saprophyticus ATCC 15305*	*S. aureus* A222	*S. sobrinus* OMZ176	*Total strains inhibited*
K12	**0.5%**	**0.5%**	18	8.5	0	0	0	9	nr	3/6
	0.5%	**2.5%**	6	0	0	0	0	0	0	1/7
	1.25%	**1.25%**	18	0	0	4	0	9	0	3/7
	1.25%	**1.7%**	18	4.5	0	8	0	0	0	3/7
	1.25%	**2.5%**	13	12.5	0	15	0	0	0	3/7
	1.7%	**1.25%**	13	46.5	0	0	0	10	0	3/7
	1.7%	**1.7%**	10	5.5	0	2	0	0	0	3/7
	2.5%	**0.5%**	18	4.5	8	6	4	13	0	6/7
	2.5%	**1.25%**	8	1.5	0	0	0	0	0	2/7
	2.5%	**2.5%**	19	5.5	0	0	0	0	0	2/7
M18	**0.5%**	**0.5%**	0	25.5	0	12	3	0	0	3/7
	0.5%	**2.5%**	0	7.5	0	0	0	0	0	1/7
	1.25%	**1.25%**	0	12.5	0	0	0	0	0	1/7
	1.25%	**1.7%**	0	5.5	0	0	0	0	0	1/7
	1.25%	**2.5%**	0	5.5	0	0	0	0	0	1/7
	1.7%	**1.25%**	0	13.5	0	18	0	0	0	2/7
	1.7%	**1.7%**	0	3.5	0	0	0	0	0	1/7
	2.5%	**0.5%**	0	9.5	0	4	0	0	0	2/7
	2.5%	**1.25%**	0	8.5	0	6	0	0	0	2/7
	2.5%	**2.5%**	0	6.5	0	0	0	0	0	1/7

Example 8: Activity of raffinose (2.5%) with K12 or M18 compared Trimix (mixture of equal weight percentage concentrations of the three saccharides) and individual saccharides

This experiment shows that the inhibitory spectrum of K12 or M18 was found to be extended to species and strains, not typically inhibited by K12 or M18. This concentration of raffinose was found to inhibit several ENTR, dental and skin pathogens and *S. salivarius* strains

[00391] The method used was the same as Example 4.

[00392] Results **Figures 14-23** shows the inhibitory effects of K12 or M18 in against ENTR, dental and skin pathogens and other *S. salivarius* strains sensitive to bacteriocin producing *S. salivarius* K12 or M18.

Example 9: Raffinose promotes inhibitory activity of K12 and M18 when K12 and M18 is sourced from a different fermentation process and contains a different lyoprotectant mix i.e. dairy free

[00393] This experiment shows that raffinose enhances the inhibitory effect of K12 and M18 irrespective of the source of K12 in ingredient supplier and the different lyoprotectant matrix that houses K12 or M18.

[00394] The method used is described in example 4. The pH of the producer streak was adjusted after the growth of producer to change the acidic conditions for all test plates.

[00395] The enhanced inhibitory action of dairy free K12 with 2.5% w/w raffinose over an equal weight percentage of saccharides either as a trimix or individual saccharides was seen against representative microorganisms from skin, dental, oral, ENTR diseases and as well as other *S. salivarius* strains **(Figure 24-25)**. The activity is normalised to baseline control.

[00396] **Conclusion:** Similar to K12 or M18, dairy free K12 or M18 also showed enhanced inhibitory activity against a variety of microorganisms in presence of raffinose and galactose. Comparatively, trimix or other saccharides did not show similar inhibitory effect.

Example 10A: Effect of raffinose and galactose on the growth and induction of inhibitory activity of K12

[00397] This example shows that raffinose and galactose do not contribute to greater cell count of K12 but still enhance antimicrobial effect.

[00398] **Method:** Growth curves were conducted using K12 raw ingredient as a starting culture of $1x10^5$ CFU/ml in M17 broth (control) [BD Difco # 218561] or M17 broth (test) supplemented with either 2.5% raffinose [D-(+)-raffinose pentahydrate - Sigma #R0250], 2.5% Trimix, 0.83% or 2.5% galactose [D-galactose – BD Difco #216310], 0.83% or 2.5% glucose [D(+)-Glucose – monohydrate – Applichem #A3617]or 0.83% or 2.5% fructose [D(-)Fructose – Sigma #F3510](all w/v). Broth cultures were then incubated at 37ºC,5% CO_2 in air and samples were taken and analysed at following timepoints of 0, 6 and 18 hours for cell count and optical density using a spectrophotometer (Optical Density (OD) of 600nm). To determine cell count, 1:10 serial dilutions of the culture samples were prepared in Phosphate Buffered Saline (PBS Dulbecco A – Oxoid #BR0014G) and then 20µl spots of each dilution were spotted in triplicate onto CABK12 agar plates. These were then incubated at 37 ºC, 5% CO_2 in air for 18h. The cell count for each culture sample at the different timepoints was then calculated from the number of colonies grown at each of the 1:10 dilutions.

[00399] **Results:**

- All saccharide combinations resulted in a similar cell counts for K12, thus they did not result in any substantial difference except for glucose which gave the highest cell count **(Figure 26)**.

- But despite this, glucose did not result in any greater inhibitory activity (likely due to catabolite repression).

- Surprisingly K12 grown in presence of raffinose and galactose shows greater inhibitory activity against *S. pyogenes* strains **(Figure 27)** or other pathogens **(Figure 28)**.

- Trimix or individual saccharides of raffinose as supplied separately did not result in any greater increase in inhibitory activity compared to the control.

Example 10B: Effect of raffinose and galactose on the growth and induction of inhibitory activity of M18

[00400] This example shows that raffinose and galactose do not contribute to greater cell count of *S. salivarius* M18 but still enhance antimicrobial effect.

[00401] **Method:** Growth curves were conducted using M18 raw ingredient as a starting culture of $1x10^5$ CFU/ml in M17 broth (control) [BD Difco # 218561] or M17 broth (test) supplemented with either 2.5% raffinose [D-(+)-raffinose pentahydrate - Sigma #R0250], 2.5% Trimix, 0.83% or 2.5% galactose [D-galactose – BD Difco #216310], 0.83% or 2.5% glucose [D(+)-Glucose – monohydrate – Applichem #A3617]or 0.83% or 2.5% fructose [D(-)Fructose – Sigma #F3510](all w/v). Broth cultures were then incubated at 37°C in 5% CO_2 and samples were taken and analysed at following timepoints of 0, 6 and 18 hours for cell count. To determine cell count, 1:10 serial dilutions of the culture samples were prepared in Phosphate Buffered Saline (PBS Dulbecco A – Oxoid #BR0014G) and then 20µl spots of each dilution were spotted in triplicate onto CABK12 agar plates. These were then incubated at 37 °C with 5% CO_2 for 18h. The cell count for each culture sample at the different timepoints was then calculated from the number of colonies grown at each of the 1:10 dilutions.

[00402] Results**:**

- All saccharide combinations resulted in a similar cell counts for M18 **(Figure 28A)**.

- Surprisingly M18 grown in presence of raffinose, and galactose shows greater inhibitory activity against *S. pyogenes* strain as well as other microorganisms **(Figure 28B)**.

- Trimix or individual saccharides of raffinose as supplied separately did not result in any greater increase in inhibitory activity compared to the control.

Example 11: Cell morphology of K12 and M18 raw ingredient product grown either with and without raffinose or a trimix of saccharides galactose, glucose or fructose, or the individual saccharides

[00403] The aim of this experiment was to determine whether *S. salivarius* K12 and M18 raw ingredient (-) produces sticky mucoid like colonies (compared to control). when grown on either CABCa control plates or CABCa test plates supplemented with 2.5%

raffinose or 2.5% trimix (0.83% of each of saccharides: galactose, glucose and fructose) or 0.83% of the individual saccharides.

[00404] *S. salivarius* K12 and M18 sourced from dairy free raw ingredient was also analysed for morphology changes when grown on either CABCa control plates or CABCa test plates supplemented with 2.5% raffinose or 2.5% trimix (0.83% of each of saccharides: galactose, glucose and fructose).

[00405] **Method:** Columbia Blood Agar + 0.5 % $CaCO_3$ (CABCa– To a 180ml bottle, 0.9g of Calcium carbonate ($CaCO_3$) PanReac Applichem # 141212,1210 was added to the bottle of agar by preparing a well in the agar using a scalpel and tipping in the calcium carbonate and covering with the cut-out agar. The agar was then melted in the autoclave for 110°C/10mins before cooling and mixing well before pipetting 20ml into petri dishes.

[00406] CABCa + raffinose (2.5%w/v) - To a 180ml bottle, 0.9g of $CaCO_3$and 4.5g (2.5%) of D-(+)-raffinose pentahydrate (Sigma # R0250) was added to the bottle of agar by preparing a well in the agar using a scalpel and tipping in the calcium carbonate and covering with the cut-out agar. The agar was then melted in the autoclave for 110°C/10mins before cooling and mixing well before pipetting 20ml into petri dishes.

[00407] CABCa + Trimix (mixture of equal weight percentage concentrations of the three saccharides). - To a 180ml bottle, 0.9g of $CaCO_3$ and 1.5g (0.83%) of each saccharide: D- galactose, D(+)-Glucose and D(-)Fructose was added to the bottle of agar by preparing a well in the agar using a scalpel and tipping in the calcium carbonate and carbohydrate and covering with the cut-out agar. The agar was then melted in the autoclave for 110°C/10mins before cooling and mixing well before pipetting 20ml into petri dishes.

[00408] **D-(+)-raffinose pentahydrate** - Sigma #R0250 mol. wt = 594.51 g/mol

[00409] **D-galactose** – BD Difco #216310 mol wt = 180.16 g/mol

[00410] **D(+)-Glucose – monohydrate** – Applichem #A3617,1000 mol.wt = 198.17 g/mol

[00411] **D(-)Fructose** – Sigma -#F3510 mol.wt = 180.16 g/mol

[00412] CABCa agar test plates were prepared, supplemented with either the individual saccharides; galactose, glucose and fructose or a trimix of the saccharides at a concentration of 0.83%w/v. Also, CABCa test plates supplemented with raffinose at

2.5%w/v were prepared. All test plates were supplemented with 0.5% calcium carbonate. Also, CABCa agar supplemented with 0.5%w/v calcium carbonate plates were prepared as control plates.

[00413] Freeze dried raw ingredient: - K12 –1.94 x 10^{11} cfu/g, -dairy free K12 –2.4 x 10^{11} cfu/g, M18 –2.2 x 10^{11} cfu/g, dairy free M18 –4.2 x 10^{11} cfu/g.

[00414] Diluent: Todd Hewitt Broth (THB) – 30g Todd Hewitt Broth Powder (BD Difco #279240) + Distilled Water (1000ml) autoclaved for 121°C/15mins.

[00415] Producer preparation: 1g of raw ingredient was added to 9ml THB in a small stomacher bag giving a 1:10 dilution approx. 1x10^{10} cfu/ml. This was then mixed in the stomacher machine for 5mins. Then 1:10 serial dilutions were carried out in THB down to 10^{-4}. cfu/ml.

[00416] Spread plate: 20µl volumes of the 10^{-4} cfu/ml dilution of raw ingredient were spread onto either CABCa control or CABCa test plates. These were then incubated for 18 hours at 37 °C with 5% CO_2. The plates were then visualised and photographed to assess morphology changes.

[00417] Deferred assay plate: These were prepared as before by dispensing 100 µl of the 10^{-3} dilution of raw ingredient as droplets in a vertical line down the middle of the test CABCa plates supplemented with or without saccharides and then spread as 1.2cm streak down the middle of the plate using a cutdown plastic spreader. Plates were incubated for 18 h at 37 °C with 5% CO_2.

[00418] The raw ingredient producer streak was photographed then removed using a glass slide to visualise the bacterial mass and morphology.

[00419] **Results:** Spread plates: When *S. salivarius* K12 and M18 are grown on 2.5%w/v raffinose, larger mucoid colonies are seen than when grown on CABCA control plates. K12 and M18 colonies on CABCa test plates supplemented with either Trimix (mixture of equal weight percentage concentrations of the three saccharides) or individual saccharides, were not mucoid looking (Figure 29).

[00420] Magnified photos of K12 and M18 colonies on CABCa agar and CABCa agar supplemented with 2.5% w/v raffinose: Compared to control, *S. salivarius* K12 or M18 (dairy or dairy free) visually appear as large sticky mucoid like colonies. This effect was not observed with or 2.5%w/v trimix (0.83%w/v mixture of equal weight percentage

concentrations of the three saccharides) and or 0.83%w/v of the individual saccharides (Figure 30).

[00421] Photos of K12 producer streaks and glass slides showing bacterial growth. Dotted black ovals visually shows amount of mucous produced (Figure 31).

[00422] Photos of M18 producer streaks and glass slides showing bacterial growth. Dotted black ovals visually shows amount of mucous produced (Figure 31).

[00423] K12 and M18 producer streaks also look very mucoid when they are grown on CABCa supplemented with 2.5%w/v raffinose (Figure 31).

Example 12: induction of antimicrobial effect (inhibition) varies by *S. salivarius* strain

[00424] **Aim**: to determine whether the induction of antimicrobial effect in the presence of supplemental saccharides is an inherent property of all *S. salivarius* strains.

[00425] **Method**: Preparation of solid culture media - CABCa agar plates were prepared with or without either 2.5% w/w raffinose or 0.5% w/w galactose by the addition of 0.5% (w/v) calcium carbonate to solid CAB agar in bottles and then melted by autoclaving at 110°C for 10mins. Once cooled, filter sterilised solutions of the saccharides or distilled water were added and mixed and then 20ml pipetted into petri dishes.

[00426] Preparing *S. salivarius* producer suspension, about 6 colonies of each *S. salivarius* strain were added into separate tubes containing 1ml THB and mixed well.

[00427] Deferred antagonism assay - 100 µl of *S. salivarius* producer suspensions was dispensed onto CABCa plates supplemented with or without raffinose or galactose as droplets in a vertical line down the middle of the test plate. This suspension was then spread as 1.2cm streak down the middle of the plate using a cutdown plastic spreader. Plates were incubated for 18 hours at 37 °C,5% CO_2 in air.

[00428] After incubation, the bacterial growth was then removed from the agar plate using a sterile cotton swab. The pH of the producer streak was then adjusted by placing a 1cm wide strip of filter paper soaked in 0.5M sodium carbonate pH 11 onto the agar plate to buffer the acid and adjust the pH up to around pH 6.5 - 7.5.

[00429] Plates were surface sterilised with chloroform vapour for 30 minutes, followed by air drying for 30 minutes.

[00430] Bacterial indicator suspensions were prepared by adding 3-9 colonies (depending on the size) of each strain into separate tubes containing 3ml THB. These suspensions were then swabbed across the agar plate perpendicular to the producer streak and then the agar plate was incubated for a further 18 hours at 37 °C,5% CO_2 in air.

[00431] **Results:** A range of *S. salivarius* strains were assayed including strains obtained from ATCC. When grown in presence of a specific concentration of 2.5% w/w of raffinose (Figure 32) or 0.5% w/w of galactose (Figure 33), almost all bar one strain did not show inhibitory activity against pathogens implicated in the ENT and Skin infections. The exception was *S. salivarius* strain ATCC 7073, which was the only other strain apart from K12 and M18 to have antimicrobial activity when supplemented with 2.5% w/w raffinose.

[00432] Conclusion: Induction of inhibitory activity in *S. salivarius* by raffinose or galactose is not an inherent property of *S. salivarius*. Supplementing other *S. salivarius* strains with galactose does not induce the same inhibitory effect as was observed for K12 and M18. With the exception of *S. salivarius* strain ATCC 7073, supplementing other *S. salivarius* strains with raffinose does not induce same inhibitory effect as was observed for K12 and M18.

Example 13: Induction of antimicrobial activity in K12 or M18 against gram-negative pathogens, including those implicated in causing halitosis

[00433] The aim of this experiment was to investigate the induction of inhibitory activity by galactose or raffinose in K12 or M18 against gram-negative bacteria *F. nucleatum*, *P. gingivalis* and *P. intermedia* which are not inhibited by K12 or M18 alone, and can cause halitosis and other dental infections.

[00434] **Method**: CABCa agar plates were prepared with or without 2.5% w/w raffinose, 0.5% w/w galactose or a combination of both saccharides by the addition of 0.5% (w/v) calcium carbonate to solid CAB agar in bottles and then melted by autoclaving at 110°C for 10mins. Once cooled, filter sterilised solutions of the saccharides or distilled water were added and mixed and then 20ml pipetted into petri dishes.

[00435] A deferred antagonism assay was conducted using K12 or M18 raw ingredient suspended in THB. This was spread as a 1.2cm streak containing approximately 1-2x10^6 cfu down the middle of a CABCa control or CABCa galactose supplemented test plate. After 18hrs growth at 37°C, 5% CO_2 in air, bacterial growth was removed, and the pH of the agar in the initial streak was measured and adjusted to a pH of

6.5 -7.5 using 0.5M sodium carbonate pH11 before sterilizing the agar surface using chloroform vapor. Bacterial test strains were suspended in THB and swabbed across the plates perpendicular to the initial streak and then the plates were incubated at 37°C in an anaerobic jar containing an anaeroGen sachet for 4 days. Zones of inhibition for each test strain were then measured (mm).

[00436] **Result:** K12 was found to inhibit a variety of strains of halitosis-causing gram-negative bacteria when supplemented with either 2.5%w/w raffinose or a combination of 2.5%w/w raffinose and 0.5%w/w galactose (Figure 34). A solution of 0.5% w/w galactose on its own did not induce antimicrobial activity in K12 against any of the bacterial species associated with halitosis tested.

[00437] M18 was also found to inhibit some strains of halitosis-causing bacteria when supplemented with either 2.5%w/w raffinose or a combination of 2.5%w/w raffinose and 0.5%w/w galactose (Figure 35).

Example 14: Inhibitory effect when galactose and raffinose are supplemented to K12 and M18 freeze dried raw ingredient powder

[00438] **Aim**: To determine the inhibitory activity of K12 and /or M18 raw ingredient powder in presence of raffinose and /or galactose.

[00439] Method: The amount of raffinose and galactose used was calculated based on the volume of the area of the producer streak, (which was calculated to be 4.25g) to allow for the absorption of the saccharides into the agar. Based on this, amount of 0.021g (i.e.0.5% w/w galactose of 4.25g agar volume) of galactose, 0.11g (i.e 2.5% w/w) of raffinose and the combination of galactose (0.5% w/w) and raffinose (2.5% w/w) (0.13g total) were weighed into sterile containers. K12 and/or M18 raw ingredient were suspended and diluted with sterile distilled water to a concentration of 1- $2x10^6$ cfu/100µl. Then, 100µl of K12 only, M18 only, and K12 + M18 suspensions were spread as a 1cm streak down the middle of a CABCa agar plate using a sterile spreader. In addition, 100µl suspensions of K12 only, M18 only and K12+M18 were mixed using a sterile stirring rod with the galactose and/or raffinose powders weighed above. The total volume of each mixture was pipetted using a large bore tip down the centre of a CABCa agar plate and spread as a 1cm streak down the middle with a sterile spreader. All plates were incubated lid upwards for 18 h at 37°C, 5% CO_2 in air. Bacterial growth was then removed using a microscope slide, and the pH of the agar in the producer streak area was measured and adjusted to pH 6.5-7.5 using 0.5M sodium carbonate (pH 11) before surface sterilizing the

plates with chloroform vapour. Indicator bacterial test strains were suspended in 3ml sterile THB and swabbed across the plates perpendicular to the producer steak area. The plates were incubated for a further 18-24 h at 37°C, 5% CO_2 in air. Zones of inhibition for each test organism were then measured in mm using a ruler. The amount of raffinose and galactose to be used was calculated based on the volume of the area of the producer streak, (which was calculated to be 4.25g) to allow for absorption into the agar.

[00440] **Results**: Both galactose (0.5%w/w) and raffinose (2.5%w/w) and their combinations were found to have induced the inhibitory activity in the freeze-dried raw ingredient powder of K12 (Figure 36) or M18 (Figure 37) or K12 and M18 combination (Figure 38).

Example 15: Effect of raffinose and galactose on the induction of inhibitory activity in a commercial powder formulation (Daily Defence Junior) containing *S. salivarius* K12

[00441] Aim: To compare the inhibitory effect of *S. salivarius* K12 in a commercial powder formulation Daily Defence Junior (Composition: *S. salivarius* K12 (1.25×10^9 cfu/0.8g), Isomalt, Maltodextrin, Vanilla flavour) with the supplemental saccharides raffinose and galactose added.

[00442] **Method**: Formulations for testing were prepared as follows:

1. Control: Commercial powder formulation (Daily Defence Junior) containing *S. salivarius* K12
2. Galactose 0.5% w/w was added to the commercial powder and mixed thoroughly to achieve a uniform mixture.
3. Raffinose 2.5% w/w was added to the commercial powder and mixed thoroughly to achieve a uniform mixture.

[00443] The following method was used to measure the inhibitory effect of *S. salivarius* K12 in the context of the powder formulations. Exactly 40 mL of 50°C molten CAB agar containing 0.5% calcium carbonate (CABCa) was poured in agar plates (120 x 120mm). Once the agar was set upon cooling, it was split in the middle and half of the agar gel was removed. The other half was left on the plate as Blank agar marked as side "A". Approximately, 0.8g of each combination (DDJ powder with (1) galactose, (2) raffinose or (3) combination of raffinose and galactose) was mixed with 1 mL of sterile distilled water and vortexed to produce a homogeneous suspension. 100 µl of the suspension was reserved for spreading on the surface of agar (producer side B). The remaining suspension was then mixed with 20 mL of molten CABCa agar and the mixture was poured into the empty half of the agar plate to form producer side "B". The 100 µL of

the reserved suspension was spread over the surface on side B and the plates were incubated for 18 h, 37 °C, 5% CO_2 in air. Post incubation, bacterial growth on the producer side B was removed. The pH of the producer side was measured and adjusted to 6.5-7.5 by soaking into the agar a solution of 0.5 M sodium carbonate pH 11. The plate surface was sterilized with chloroform vapours. Bacterial indicator strains were suspended in THB and were streaked across the agar plates from left (blank agar side A) to right (producer side B) using sterilised cotton swabs. Plates were again incubated for 18 h at 37°C, 5% CO_2 in air. The zone of inhibition of each indicator strain was measured (in mm) using a ruler. Results were also read using a macroscope to establish presence of small colonies or complete inhibition zones.

[00444] **Results**: Figure 39 shows that the inhibitory activity of *S. salivarius* K12 was increased in the powders containing raffinose (2.5%w/w), galactose (0.5%w/w) or their combination (raffinose 2.5%w/w + galactose 0.5%w/w) compared to the commercial powder containing K12 on its own (control).

Example 16: Properties of formulation from US 20190343899 A1

[00445] **Aim**: To determine the manufacturing conditions for the prior art formulation from US published patent application 20190343899.

[00446] **Method**: Formulation was prepared following the directions in example 1 of prior art D1. Briefly, liquid ingredients were mixed together and added slowly to the solid ingredients and heated to around 100°C on a hotplate until melted. The mixture was then allowed to cool down until it completely solidified at approximately 60°C.

[00447] **Results**: The formulation described in Example 1 of US 20190343899 was prepared by melting the ingredients to prepare a formulation having the consistency of hard candy or toffee. A high temperature (around 100 °C) was required to melt the ingredients. Due to high heat, *S .salivarius* could not be added to the formulation, as temperature above 50°C is detrimental to the probiotic. For this reason it was not possible to add a probiotic to the formulation at the melt stage (above approximately 60°C), particularly a heat-sensitive probiotic such as *S. salivarius*, without total loss of probiotic.

[00448] During cooling, the consistency of the formulation which would have enabled admixture of probiotics into the formulation with sufficient homogeneity was only maintained at a temperature of 60 °C or higher. This temperature was still too high to add the probiotic without causing cell death which occurs from around 50 °C or higher.

Example 17: Comparative example - formulation from WO 2017129639 A1

[00449] **Method:** A powdered infant nutrition product (infant formula) of similar composition to that of Example 1 of the publication WO 2017129639 was purchased (Similac 360 Total Care (Abbott Global). This product contains vitamins, minerals, lactose, 5 human oligosaccharides and whole milk powder.

[00450] To determine the effect of supplemental saccharides on the induction of inhibitory activity in *S. salivarius* K12 in the infant formula, *S. salivarius* K12 and supplemental saccharides were added to the infant formula as follows.

1. Control: *S. salivarius* K12 was added to the infant formula powder and mixed thoroughly to achieve a uniform mixture containing approx. $1.25x10^9$ cfu/g *S. salivarius* K12.
2. Galactose 0.5% w/w: *S. salivarius* K12 was added to the infant formula powder and 0.5% w/w galactose and mixed thoroughly to achieve a uniform mixture containing approx. $1.25x10^9$ cfu/g *S. salivarius* K12.
3. Raffinose 2.5% w/w: *S. salivarius* K12 was added to the infant formula powder and 2.5% w/w raffinose and mixed thoroughly to achieve a uniform mixture containing approx. $1.25x10^9$ cfu/g *S. salivarius* K12.

[00451] Measurement of induction of inhibitory activity was conducted in the same way as for Example 15.

[00452] Results: Surprisingly, the addition of galactose or raffinose to the control formulation of Example 17 showed a reduction in inhibitory activity compared to the control (Table 10).

Example 18: Comparison of antimicrobial properties of powder formulations

[00453] **Method:** The antimicrobial activity of a number of compositions was tested, as follows.

1. Control formulation of Example 17;
2. Whole milk Powder: *S. salivarius* K12 was added to the whole milk powder (Anchor Blue™ Milk powder, Anchor, NZ) and mixed thoroughly to achieve a uniform mixture containing approx. $1.25x10^9$ cfu/g *S. salivarius* K12

[00454] Measurement of induction of inhibitory activity of the powder formulations was conducted in the same way as for Example 15.

[00455] **Results**: Surprisingly these two formulations induced none, or less significant inhibitory activity compared with the three formulations of Example 15, containing commercial *S. salivarius* K12 Daily Defence Junior powder product which had been supplemented with raffinose, galactose and combination thereof (Figure 40).

Table 10: **Effect of Raffinose and Galactose on the inhibitory activity of S. salivarius K12 in an infant formula against various pathogens.**

	% change in zone size (in mm) normalised to control																		
	***S. constellatus* T29**	***S. pyogenes* 71-698**	***S. mutans FW75***	***S. mutans OMZ175***	***S. pneumoniae D39***	***S. pneumoniae PK8***	***S. aureus #19***	***S. aureus* A222**	***S. cohnii* ATCC 29974**	***S. simulans* ATCC 27848**	***S. agalactiae* ATCC 12386**	***A. viscosus* ATCC 15987**	***C. auris* ATCC 51966**	***S. sanguis K11***	***S. sobrinus OMZ 176***	***S. sobrinus* ATCC 27351**	***S. dysgalactiae Bris 2***	***S. dysgalactiae T277***	***S. dysgalactiae* T-148**	***S. cohnii***
A	**-12**	**-3**	13	0	**-4**	**-46**	**-60**	**-60**	100	300	0	0	**-14**	**-10**	13	4	17	75	21	**-42**
B	**-20**	0	0	**-8**	**-4**	**-35**	**-100**	**-100**	**-100**	**-100**	0	**-20**	**-20**	**-31**	**-13**	**-13**	**-17**	**-17**	**-31**	**-100**

A = Infant formula Formulation 2 of Example 17 containing Galactose 0.5%w/w, B = Infant formula Formulation 3 of Example 17 containing Raffinose 2.5% w/w , **-** sign denotes reduction in activity compared to control Formulation 1 of Example 17

Example 19: Upregulation of beneficial genes

[00456] **Aim**: To investigate upregulation of genes encoding antimicrobial molecules, and any other beneficial genes within the *S. salivarius* K12 genome which may cause the increased antimicrobial potency in the presence of galactose or raffinose.

[00457] **Method**: CABCa agar plates were prepared either with or without 2.5% w/w raffinose, 0.5% w/w galactose, combined 2.5% w/w raffinose and 0.5% w/w galactose, 0.5% w/w glucose or 2.5% w/w glucose by the addition of 0.5% (w/v) calcium carbonate to solid CAB agar in bottles and then melted by autoclaving at 110°C for 10mins. Once cooled, filter sterilised solutions of the saccharides or distilled water were added and mixed and then 20ml pipetted into petri dishes.

[00458] A *S. salivarius* K12 raw ingredient suspension was prepared in PBS containing approx. $1x10^8$cfu/ml. 100µl of this suspension was spread evenly onto control and test agar plates. Plates were incubated at 37°C with 5% CO_2 for approx, 19hrs.

[00459] Bacterial growth from each plate was collected using a sterile cotton swab and resuspended into 1ml PBS in a screw capped tube. The bacterial cells were then pelleted by centrifugation at 13,000rpm for 1 minute at 4°C. The supernatant was removed, and the cell pellet was resuspended in 1ml of TRIzol reagent. The bacterial cell suspension was transferred into a 2ml screw-capped tube containing 0.1 mm Zirconia/silicon beads. These tubes were then vortexed for 5 minutes at the highest speed to bead beat the bacterial cells to lyse them. The tubes were placed on ice for 1 minute and then re-bead beated again on the vortex for 5 minutes. The tubes were then frozen at -20°C.

[00460] The lysed bacterial suspension was thawed and 0.2ml chloroform was added to each tube. The tubes were incubated for 2-3 minutes and kept on ice and also mixed by hand by inverting the tubes frequently. The beads were then left to settle, and then the upper liquid suspension was transferred into the phase-maker tubes. The tubes were incubated on ice for 5 minutes whilst mixing by hand by inverting the tubes frequently. The tubes were then centrifuged at 12,000rpm at 4°C for 15 minutes to separate into a lower (red) phenol-chloroform phase, interphase and upper (clear) aqueous phase.

[00461] 560µl of the clear upper aqueous phase was transferred into a new Eppendorf tube and then frozen again at -20°C.

[00462] The laboratory bench and other equipment used for the RNA extraction was treated with RNaseZap to remove any potential RNases.

[00463] RNA was extracted from the TRIzol thawed suspensions using the Thermofisher PureLink RNA mini kit following the manufactures' instructions for extracting RNA from TRIzol samples as follows:

[00464] Added 600µl of 70% ethanol to each tube of thawed lysed bacterial suspension. Transferred approx. 600 µL of the sample to a spin cartridge within a Collection Tube.

[00465] Centrifuged at 12,000 × g for 15 seconds at room temperature. Discarded the flow-through and reinserted the spin cartridge into the same collection tube. Added the final 600µl of the sample and centrifuged the spin column again. Discarded the flow-through and reinserted the spin cartridge into the same collection tube.

[00466] Added 700 µL Wash Buffer I to the spin cartridge. Centrifuged at 12,000 × g for 15 seconds at room temperature. Discarded the flow-through and the collection tube. Inserted the spin cartridge into a new collection tube.

[00467] Added 500 µL Wash Buffer II to the spin cartridge.

[00468] Centrifuged at 12,000 × g for 15 seconds at room temperature. Discarded the flow-through and reinserted the spin cartridge into the same collection tube. Added another 500 µL Wash Buffer II to the spin cartridge and centrifuged at 12,000 × g for 1 minute at room temperature to dry the membrane. Discarded the collection tube and insert the spin cartridge into a fresh eppendorf tube.

[00469] Added 100 µL RNase–Free Water to the center of the spin cartridge. Incubated at room temperature for 1 minute. Centrifuged the spin cartridge with the eppendorf tube for 2 minutes at ≥12,000 × g at room temperature to elute the RNA.

[00470] The concentration of the eluted RNA was measured using a Nanodrop.

[00471] The extracted RNA was then DNase treated to remove any contaminating DNA using the Thermo Fisher TURBO-DNA Free kit as follows:

[00472] Prepared a 50µl reaction containing less than 50µg RNA. The same concentration of RNA was added to the reaction for each sample, calculated from the Nanodrop RNA concentration to standardize the amount of RNA across all the samples.

Mixed up the reactions in Eppendorf tubes with the volume of RNA required, 5 µl Turbo DNase buffer, 2 µl Turbo DNase and then made up the volume to 50 µl with nuclease free water whilst keeping all the reagents on ice. A no RNA control sample was prepared to use as a blank when measuring the concentration of the DNase treated RNA samples using the nanodrop. Eppendorf tubes were incubated at 37°C for 30 minutes. The reaction was then inactivated by the addition of 10 µl DNase inactivation reagent and mixed well. Eppendorf tubes were incubated at room temperature for 5 mins, inverted 2-3 times to mix reagents during incubation. Samples were the centrifuged at 10,000 x g for 1.5 minutes. The supernatant was then transferred into a fresh Eppendorf tube which was also centrifuged, and the supernatant was transferred again into a fresh Eppendorf tube. The concentration of the DNase treated RNA samples was then measured using the nanodrop, using the no-RNA control sample as a blank.

[00473] DNase treated RNA samples were then checked for DNA contamination by PCR, using SalB primers, which would produce a DNA band of approximately 500bp in size.

[00474] PCR reactions were prepared in 0.2ml PCR tubes for each DNase treated RNA sample, a positive control sample of extracted DNA from *S. salivarius* K12 and a negative control sample containing nuclease free water only. 25 µl reactions were prepared by mixing 12.5 µl GoTaq G2 Hot start green Master mix, 1 µl SalB forward primer, 1 µl SalB reverse primer, 1 µl of either RNA or DNA sample and nuclease free water up to a volume of 25 µl.

[00475] PCR amplification consisted of initial denaturation 15min at 94°C, followed by 30 cycles of: Denaturation – 30 secs at 95°C; Annealing – 30 secs at 40°C; Extension – 30 secs at 73°C. After the 30 cycles, another 2min at 92°C.

[00476] After PCR amplification a 0.5cm thick 2% agarose / 1x TAE gel containing 1X SYBR safe DNA gel stain and a 1.5mm width comb was loaded with either 10 µl of each DNase treated RNA sample or 5 µl of the AccuRuler 1kb DNA RTU ladder into a well to determine the band size of any visualised bands.

[00477] No DNA bands detected in the DNase treated samples confirming that any DNA contamination was removed.

[00478] Next RNA samples were converted to cDNA using the superscript IV vilo master mix, following the manufactures instructions as follows:

[00479] Prepared 20 µl reactions in 0.2ml PCR tubes containing up to 2.5µg RNA, normalised the amount of RNA added, so that all the samples had the same concentration of RNA in the tubes. To each tube 4 µl of superscript IV vilo master mix was added and the volume was made up to 20µl with nuclease free water. A duplicate set of reactions were prepared containing the same concentration of RNA for each sample but with the addition of 4µl of superscript IV vilo No RT control and the volume was also made up to 20µl with nuclease free water.

[00480] PCR tubes were then placed in an PCR machine to conduct the following incubations: 10 minutes at 25°C (primer annealing) ; 10 minutes at 50 °C (reverse transcribe RNA) ; 5 minutes at 85 °C (inactivate enzyme).

[00481] The cDNA samples from K12 raw ingredient grown on the CABCa control plates and CABCa supplemented with the various saccharides were then analysed by qPCR for the levels of gene expression of the following genes: *salA*, *salB*, *salQ* and *ureC*.

[00482] 10 µl qPCR reactions were prepared containing 2 µl of cDNA, 5 µl SYBR green master mix, 0.5 µl forward primer, 0.5 µl reverse primer and nuclease free water up to 10 µl. The dilution of cDNA in each qPCR was optimised with each primer set.

[00483] qPCR method consisted of the following cycles: Hold Stage: 2 mins at 50°C followed by 10 mins at 95°C; PCR Stage: 15 secs at 95°C followed by 1min at 60°C; Melt Curve Stage: 15 secs at 95°C followed by 1 min at 60°C followed by 15 secs at 95°C.

[00484] To analyse the relative expression levels the $2^{-\Delta\Delta Ct}$ method was used to determine the relative fold gene expression level comparing the different sugars to a CABCa plate control. The reference gene used for this analysis was *gyrA*.

[00485] **Results**: Both *salA* and *salB* genes were upregulated in K12 were relative to a no sugar control when either 2.5%w/w raffinose, 0.5%w/w galactose or the combination of both was added (Figure 41 and 42). Interestingly 0.5%w/w galactose seemed to cause the greatest increase in gene expression (~12000 fold) of *salA* and (641 fold) of *salB*, followed by 2.5%w/w raffinose (~5000 and 241 fold) and then the combination (~2400 and 160 fold). The upregulation of theses gene did not occur to the same extent when a control sugar glucose was added in either 0.5%w/w (~50 and 23 fold) or 2.5%w/w (5.2 and 0.8 fold) concentrations.

[00486] Additionally, *salQ* was upregulated relative to a no sugar control for when either 2.5%w/w raffinose (93 fold), 0.5%w/w galactose (205 fold) or the combination of

both (45 fold) was added (Figure 43). However, *salQ* also appears to be upregulated when 2.5%w/w glucose (81 fold) is added indicating that this may be a general sugar effect.

[00487] When either 2.5%w/w raffinose, 0.5%w/w galactose or the combination of both was added to the medium there was an upregulation in urease (*ureC*) expressed with increases of 234 fold, 224 fold and 191 fold respectively (Figure 44). 0.5%w/w and 2.5%w/w glucose also gave moderate increases in urease expression of 22 fold and 96 fold respectively.

Example 20: Change in the level of K12 colonisation in the oral cavity of healthy human volunteers

[00488] **Aim**: To determine if the addition of galactose and/or raffinose to a commercial *S. salivarius* K12 lozenge formulation (Throat Guard Pro, Blis Technologies (NZ)) will change the colonisation level of K12 when consumed once a day for 7 days.

[00489] **Method**: A double-blind, randomized controlled colonization pilot study with no cross over was conducted in healthy human adults to evaluate the colonization efficacy of lozenges containing *S. salivarius* K12 (~2.5 Billion cfu/lozenge) without galactose or raffinose (control G1, containing *S. salivarius* K12, isomalt, tableting aids and natural flavour Blis Technologies (NZ)) and lozenges additionally containing: raffinose 2.5% w/w (G2), galactose 0.5% w/w (G3), and a combination of galactose 0.5% w/w and raffinose 2.5% w/w (G4).

[00490] Lozenges G2-G4 were prepared by blending *S. salivarius* K12, isomalt, tableting aids and natural flavour, with: raffinose 2.5%w/w (G2); galactose 0.5% (G3); and galactose 0.5% w/w and raffinose 2.5% w/w (G4). Each blend was then subjected to the tableting machine to obtain lozenges. Each of the lozenges in the four groups was formulated to contain about 2.5 billion cfu/lozenge.

[00491] Participants were enrolled if they were healthy and practice good oral hygiene, 18 – 80 years of age, not on antibiotic therapy, immunocompromised or on history of autoimmune disease, people with allergy or sensitivity to dairy. Following the inclusion criteria, a total of 20 participants were recruited and divided into 4 groups.

Study Group: K12 Lozenges (2.5B cfu/lozenge)

G1: K12 lozenges (control) (n = 5)

G2: K12 lozenges containing raffinose (2.5%w/w) (n = 5)

G3: K12 lozenges containing galactose (0.5%w/w) (n = 5)

G4: K12 lozenges containing raffinose (2.5%w/w) and galactose (0.5%w/w) (n = 5)

[00492] Participants were asked to gargle with mouthwash (only on the first night), wait 1 h and then collect saliva sample (pre-trial sample). They were then asked to slowly dissolve one lozenge in the mouth. Participants were then asked to collect further saliva samples 1 hour, 8 hours and 24 hours after taking the lozenge. Participants were then asked to take1 lozenge per night for 6 more nights, and collect a final saliva sample 48h after the last dose..

[00493] Microbial sampling and analysis: During and at the end of the trial, the tubes containing saliva samples for each time point and each participant were collected and stored in a freezer (-20 ˚C) until analysed.

[00494] Saliva samples were serially diluted (multiple repeats of 100 µL sample resuspended in 900 µL of PBS) to 10^{-4} and spread plated on Mitis-Salivarius agar plates (a *Streptococcus salivarius* selective media) using a 50 µL inoculum per plate. The plates were incubated for 24h at 37°C, 5% CO_2 in air. After incubation K12 or M18 colonies were differentiated by their inhibition activity against the specific indicator strains I1 (*Micrococcus luteus* T-18) and I3 *Streptococcus constellatus* T-29). Suspensions of the indicator strain I1 was made by the addition of 1 colony to 3ml of THB, and the I3 suspension was made by the addition of 4 colonies to 3ml of THB. The indicator strains were swabbed on to blood agar plates (sBaCa) covering the entire surface of the agar. Using a toothpick, the *S. salivarius* like colonies grown from the saliva samples on the mitis salivarius agar were spiked into the I1 then I3 pre-seeded indicator lawns and incubated for 24h at 37 ˚C, 5% CO_2 in air. Colonies with an inhibition zone for both I1 and I3 were identified as presumptive positive K12 as they indicate the activity of the *salA* and *salB* genes.

Results

[00495] Figure 45 shows that the average percentage of *S. salivarius* K12 (of total *S. salivarius*) in the saliva samples obtained from the groups of participants using the lozenges with supplemental saccharide galactose (G2), raffinose (G3) and raffinose and galactose (G4) was greater than the average percentage for the control group with *S. salivarius* K12 alone (Control G1). Thus, the presence of the supplemental saccharides enhanced the colonisation efficacy of *S. salivarius* K12 in the oral cavity.

[00496] In the supplemental saccharide groups, raffinose (G3) showed the maximum increase in colonisation followed by galactose (G2) and combination of raffinose and galactose (G4) compared to pre-trial levels for all sample points. The percentage of *S. salivarius* K12 in the total *S. salivarius* population remains higher for the raffinose group (G3). The level in the raffinose group was maintained higher then pre-trial even after the cessation of lozenge consumption, suggesting improved persistence of *S. salivarius* K12 due to the supplemental saccharides.

[00497] It is not the intention to limit the scope of the invention to the abovementioned examples only. As would be appreciated by a skilled person in the art, many variations are possible without departing from the scope of the invention as set out in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

BLIS Technologies Ltd.
c/o Dep. of Microbiology
University of Otago
P.O. Box 56
Dunedin
New Zealand

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:
Mia

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:
DSM 14685

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-12-12
(Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request
for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address: Mascheroder Weg 1b
D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

U. Weis

Date: 2001-12-14

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

University of Otago
Dept. of Microbiology
P.O. Box 56
Dunedin
New Zealand

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:
K12

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:
DSM 13084

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1999-10-06 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address: Mascheroder Weg 1b
D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 1999-11-02

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

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CLAIMS

1. A method of improving the inhibitory profile of *Streptococcus salivarius* comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

   wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

   wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

2. A method for upregulating one or more genes in *Streptococcus salivarius*, comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

   wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

   wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

3. A method according to claim 2, wherein the upregulated gene(s) encodes for a lantibiotic peptide or bacteriocin.

4. A method according to claim 3, wherein the upregulated gene(s) encodes for a Class I or Class II lantibiotic peptide or bacteriocin.

5. A method according to claim 4, wherein the lantibiotic peptide is *salA, salB*, *sal9* or a combination thereof.

6. A method according to claim 4 or 5, wherein the lantibiotic peptide is *salA, sal*B, or a combination thereof.

7. A method according to claim 4, wherein the bacteriocin is *salQ*.

8. A method according to claim 2, wherein the upregulated gene(s) encodes for a subunit of a urease protein.

9. A method according to claim 8, wherein the upregulated gene is *ureC*.

10. A method according to any one of claims 2 to 9, wherein at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 70% sequence identity to any one of SEQ ID NOs 15-22, or wherein at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 70% sequence identity to any one of SEQ ID NOs 23-30.

11. The method of claim 10, wherein at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence with at least 75% identity to any one of SEQ ID NOs 15-22, preferably at least 80%, 85%, 90%, 95%, or 99% identity to any one of SEQ ID NOs 15-22.

12. The method of claim 10 or 11, wherein at least one of the upregulated gene(s) comprises or consists of a polynucleotide sequence that encodes a polypeptide with at least 75% identity to any one of SEQ ID NOs 23-30, preferably at least 80%, 85%, 90%, 95%, or 99% identity to any one of SEQ ID NOs 23-30.

13. The method of any one of claims 10 to 12, wherein:

    a. at least one of the upregulated gene(s) is a *sal*A gene or variant thereof comprising or consisting of a polynucleotide sequence with least 70% identity to SEQ ID NO 15 or 19, or encoding a polypeptide with at least 70% identity to SEQ ID NO 23 or 27;

    b. at least one of the upregulated gene(s) is a *sal*B gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 16, or encoding a polypeptide with at least 70% identity to SEQ ID NO 24;

    c. at least one of the upregulated gene(s) is a *sal*Q gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 17 or 21, or encoding a polypeptide with at least 70% identity to SEQ ID NO 25 or 29;

    d. at least one of the upregulated gene(s) is a *sal*9 gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 20, or encoding a polypeptide with at least 70% identity to SEQ ID NO 28; and/or

e. at least one of the upregulated gene(s) is a *ure*C gene or variant thereof comprising or consisting of a polynucleotide sequence with at least 70% identity to SEQ ID NO 18 or 22, or encoding a polypeptide with at least 70% identity to SEQ ID NO 26 or 30.

14. A method for increasing production of one or more of a lantiobiotic peptide, bacteriocin or urease by *Streptococcus salivarius*, comprising formulating the *S. salivarius* in a composition comprising an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose, or raffinose, or a combination thereof.

15. A method according to claim 14, wherein the lantibiotic peptide or bacteriocin is a Class I or Class II lantibiotic peptide or bacteriocin.

16. A method according to claim 15, wherein the lantibiotic peptide is *salA, salB, sal9* or a combination thereof.

17. A method according to claim 15 or 16, wherein the lantibiotic peptide is *salA, sal*B, or a combination thereof.

18. A method according to claim 11, wherein the bacteriocin is *salQ*.

19. A method according to any one of claims 14 to 18, which increases production of a polypeptide with at least 70% sequence identity to any one of SEQ ID NOs 23-30.

20. The method of claim 19, wherein at the polypeptide has at least 75% identity to any one of SEQ ID NOs 23-30, preferably at least 80%, 85%, 90%, 95%, or 99% identity to any one of SEQ ID NOs 23-30.

21. The method of any one of claims 1-20, wherein the method increases the inhibitory profile of *S. salivarius* against skin, dental, oral, mucosal and/or ENTR microorganisms.

22. The method of claim 21, wherein the skin, oral, dental, mucosal, and/or ENTR microorganism is selected from *S. aureus* spp., *S. intermedius* spp., *S. saprophyticus* spp., *M. catarrhalis* spp., *H. influenzae* spp., *S. pyogenes* spp., *P. aeruginosa* spp., *S. mutans*

spp., *S. pneumoniae* spp., *C. acnes* spp., *C. albicans* spp. *S. sobrinus* spp., *Corynebacterim* spp., *F. nucleatum* spp., *A. actinomycetemcomitans* spp., *P. gingivalis* spp., *Tannerella forsythia* spp., *Treponema denticola* spp., *P. intermedia* spp., *Prevotella spp., A. viscosus* spp., *S. equismillis* spp., *S. dygalactiae* spp., *S. sanguis* spp., *S. cohnii* spp., *B. intermedius* spp., *A. parvulum* spp., *E. saburreum* spp., *E. sulci* spp., *P. micra* spp., *S. moorei* spp., *S. agalactiae* spp., *C. minutissimus* spp., *P. propionicus* spp., *S. agalactiae* spp., *S. dysgalactiae* spp., *S. simulans* spp., *S. xylosus* spp., Tinea pedis infection causing fungi, *S. salivarius* spp. other than K12 or M18, *L. lactis* spp., *S. epidermidis* spp., *S. constellatus* spp., *K. pneumoniae* spp., *A. baumanii* spp. or any combination of any two or more thereof.

23. The method of claim 22, wherein the microorganism is selected from *S. aureus* A222, *S. aureus* 20, *S. aureus* 14, *S. aureus* 19, *S. aureus* A504, *S. saprophyticus* ATCC 15305, *M. catarrhalis* TW1, *M. catarrhalis* TW2, *H. influenzae* TW5, *S. pyogenes* M76, *S. pyogenes* 71-698, *S. pyogenes* FF22, *S. pyogenes* 71-679, *S. pyogenes* W-1, *S. pyogenes* M17, *S. pyogenes* M57, *S. pyogenes* EMM92, *S. pyogenes* M66, *S. pyogenes* M74, *P. aeruginosa* I2, *S. mutans* OMZ175, *S. pneumoniae* D39, *L. lactis* T-21, *S. epidermidis* 11, *S. constellatus* T-29, *S. salivarius* 6, *S. salivarius* 193, *S. salivarius* 20P3, or any combination of any two or more thereof.

24. The method of any one of claims 1 to 23, wherein the composition comprises at least about 0.1% by weight of each *S. salivarius*.

25. The method of any one of claims 1 to 24, wherein the composition comprises from about 0.1 to about 20% by weight of each *S. salivarius*.

26. The method of any one of claims 1 to 25, wherein the composition comprises at least about 1×10^3 cfu/g of each *S. salivarius*.

27. The method of any one of claims 1 to 26, wherein the composition comprises from about 1×10^3 to about 1×10^{13} cfu/g of each *S. salivarius*.

28. The method of any one of claims 1 to 27, wherein the composition comprises less than about 20% by weight of each supplemental saccharide.

29. The method of any one of claims 1 to 28, wherein the composition comprises from about 0.1 to about 20% by weight of each supplemental saccharide.

30. The method of any one of claims 1 to 29, wherein the composition is formulated for oral, dental, nasal, mucosal, topical, or pulmonary administration.

31. The method of any one of claims 1 to 30, wherein the composition is formulated in a slow-release composition.

32. The method of any one of claims 1 to 31, wherein the composition is formulated into a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel spray, deodorant, serum, lotion, balm, moisturiser, pessary, or suppository.

33. A method of inhibiting a skin, dental, oral, mucosal and/or ENTR microorganism, the method comprising contacting the microorganism with a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

34. The method of claim 33, wherein the microorganism is a *Streptococcus* or *Staphylococcus* bacteria selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. pyogenes* spp., *S. pneumoniae* spp.; and the *S. salivarius* strain is K12.

35. The method of claim 33 or 34, wherein the *Streptococcus* or *Staphylococcus* bacteria is selected from *S. aureus* A222, *S. saprophyticus* ATCC 15305, *S. mutans* OMZ175, *S. constellatus* T-29, *S. pyogenes* 71-698, and *S. pneumoniae* D39; and the *S. salivarius* strain is K12.

36. The method of any one of claims 33 to 35, wherein the supplemental saccharide is raffinose and is present in the composition in an amount of 0.5 to 15%, or 1 to 12%, or 1.5 to 10%, or 2 to 7%, or 2.5 to 5% by weight.

37. The method of any one of claims 33 to 36, wherein the supplemental saccharide is galactose and is present in the composition in an amount of 0.5 to 15%, or 1 to 12%, or 1.5 to 10%, or 2 to 7%, or 2.5 to 5% by weight.

38. The method of any one of claims 33 to 37, wherein the bacteria are selected from *S. pyogenes* spp., and *S. pneumoniae* spp.; and the *S. salivarius* strain is M18.

39. The method of any one of claims 33 to 38, wherein the bacteria is selected from *S. pyogenes* 71-698, and *S. pneumoniae* D39; and the *S. salivarius* strain is M18.

40. The method of any one of claims 33 to 37, wherein the bacteria is selected from *S. constellatus*, *S. mutans, and S. saprophyticus*; and the *S. salivarius* strain is M18.

41. The method of any one of claims 33 to 37, wherein the bacteria is selected from *S. constellatus T29*, *S. mutans OMZ175, and S. saprophyticus ATCC 15305*; and the *S. salivarius* strain is M18.

42. The method of any one of claims 33 to 41, wherein the supplemental saccharide is raffinose and is present in the composition in an amount of 0.25 to 10%, or 0.5 to 8%, or 0.75 to 7%, or 1 to 6%, or 1.25 to 5% by weight.

43. The method of any one of claims 33 to 41, wherein the supplemental saccharide is galactose and is present in the composition in an amount of 0.25 to 10%, or 0.5 to 8%, or 0.75 to 7%, or 1 to 6%, or 1.25 to 5% by weight.

44. A composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide for use in improving the inhibitory profile of the *Streptococcus salivarius*,

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

45. A composition according to claim 44, comprising one or more of: galactose in an amount of 0.1 to 1%, or 0.2 to 0.8, or 0.25 to 0.75, or at 0.5% by weight, and raffinose in an amount of 0.5 to 5%, or 1 to 4, or 2 to 3, or 2.5% by weight.

46. A composition comprising *Streptococcus salivarius* K12, and raffinose in an amount of 2 to 3% by weight.

47. A composition comprising *Streptococcus salivarius* K12, and galactose in an amount of 0.25 to 0.75% by weight.

48. A composition comprising *Streptococcus salivarius* M18, and raffinose in an amount of 2 to 3% by weight.

49. A composition comprising *Streptococcus salivarius* M18, and galactose in an amount of 0.25 to 0.75% by weight.

50. A composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, and raffinose in an amount of 2 to 3% by weight.

51. A composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, and galactose in an amount of 0.25 to 0.75% by weight.

52. A composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, raffinose in an amount of 2 to 3% by weight, and galactose in an amount of 0.25 to 0.75% by weight.

53. A composition comprising *Streptococcus salivarius* K12, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

54. A composition comprising *Streptococcus salivarius* M18, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

55. A composition comprising *Streptococcus salivarius* K12, *Streptococcus salivarius* M18, raffinose in an amount of 1.2 to 2.2% by weight, and galactose in an amount of 0.7 to 1.7% by weight.

56. The composition according to any one of claims 44 to 55, further comprising one or more of a carrier; a tableting aid, including a binder or a lubricant; and a flavouring agent.

57. A therapeutic formulation comprising the composition of any one of claims 44 to 56.

58. The therapeutic formulation of claim 57, wherein the therapeutic formulation is formulated for oral, dental, nasal, mucosal, topical, or pulmonary administration.

59. The therapeutic formulation of claim 57 or 58, wherein the therapeutic formulation is a slow-release composition.

60. The therapeutic formulation of any one of claims 57 to 59, wherein the therapeutic formulation is a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel, spray, deodorant, serum, lotion, balm, moisturiser, pessary, or suppository.

61. The therapeutic formulation of claim 60, which is a powder.

62. The therapeutic formulation of claim 60, which is a lozenge.

63. A method of treating or preventing a disease or disorder comprising administering to subject in need thereof a composition of any one of claims 44 to 56, or a therapeutic formulation of any one of claims 57 to 62.

64. The method of claim 63, wherein the disease or disorder is caused by an oral, dental, mucosal, skin, or ENTR pathogen.

65. The method of claim 63 or 64, wherein the disease or disorder is caused by a pathogenic *Streptococcus* or *Staphylococcus* bacteria.

66. The method of any one of claims 63 to 65, wherein the pathogenic *Streptococcus* or *Staphylococcus* bacteria is selected from *S. aureus* spp., *S. saprophyticus* spp., *S. mutans* spp., *S. pyogenes* spp., and *S. pneumoniae* spp.

67. The method of any one of claims 63 to 66, wherein the disease or disorder is selected from otitis media, sore throat, tooth decay, acute pharyngitis, tonsillitis, pneumonia, COPD, periodontal disease, gingivitis, halitosis, dental caries, sepsis, meningitis, candidiasis (oral thrush), vaginitis, body odour, acne, actinomycosis, psoriasis, erythrasma, cellulitis, impetigo, atopic dermatitis, bacteraemia, tineas including athlete's

foot, soft tissue infections, erythema, nosocomial, erythema, SARS-CoV, influenza A, influenza B, and RSV or any combination of any two or more thereof.

68. A method of inhibiting a microorganism sensitive to Blis-producing *S. salivarius*, the method comprising administering to subject in need thereof a composition of any one of claims 33 to 37, or a therapeutic formulation of any one of claims 57 to 62.

69. The method of claim 68 wherein the microorganism sensitive to Blis-producing *S. salivarius* is selected from *S. salivarius* spp., *S. epidermidis* spp., *S. constellatus* spp., and *L. lactis* spp.

70. The method of claim 68 wherein the microorganism sensitive to Blis-producing *S. salivarius* is selected from *S. pyogenes* spp., *F. nucleatum* spp., and *P. gingivalis* spp.

71. The method of any one of claims 63 to 70, wherein the subject is a human.

72. Use of *Streptococcus salivarius* and a supplemental saccharide in the manufacture of a medicament for:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius,*

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

73. A composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide for use in:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius,*

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

74. The composition of claim 73, wherein the composition is a powder, lozenge, nasal spray, nasal gel, nasal drop, oral drop, oral gel, oral spray, inhalable, topical composition, chewable, melt, film, gummy, toothpaste, tooth-gel, varnish, mousse, mouthwash, food product (e.g. yoghurt), cream, gel, spray, deodorant, serum, lotion, balm, moisturiser, pessary, or suppository.

75. The composition of claim 74, which is a powder.

76. The composition of claim 74, which is a lozenge.

77. A composition according to any one of claims 44 to 56 or 73 to 75, wherein the composition is a cosmetic.

78. A composition according to any one of claims 44 to 56 or 73 to 76, wherein the composition is a dietary supplement.

79. A composition according to any one of claims 44 to 56 or 73 to 76, wherein the composition is a natural health product.

80. A composition according to any one of claims 44 to 56 or 73 to 76, wherein the composition is a complementary medicine.

81. A method of manufacturing a composition comprising *Streptococcus salivarius* and an effective amount of a supplemental saccharide, the method comprising:

(a) combining *Streptococcus salivarius* with supplemental saccharide, and

(b) mixing to produce a homogeneous blend:

wherein the *Streptococcus salivarius* is *Streptococcus salivarius* M18, *Streptococcus salivarius* K12, or a combination thereof, and

wherein the supplemental saccharide is galactose or raffinose or a combination thereof.

82. The method of claim 81, wherein the composition is a lozenge, and the method further comprises a step of lozenging the homogeneous blend to produce the lozenge.

83. The method of claim 81 or 82, wherein the inhibitory profile of the *S. salivarius* in the composition is improved relative to a composition lacking the supplemental saccharide.

84. The method of claim any one of claims 81 to 83, wherein the composition is for use in:

(a) the treatment or prevention of a disease or disorder, or

(b) the inhibition of a microorganism sensitive to Blis-producing *S. salivarius*.

85. Use of a composition manufactured by the method of any one of claims 81 to 84 for the treatment or prevention of a disease or disorder, or for the inhibition of a microorganism sensitive to Blis-producing *S. salivarius*.

FIGURES

Figure 1

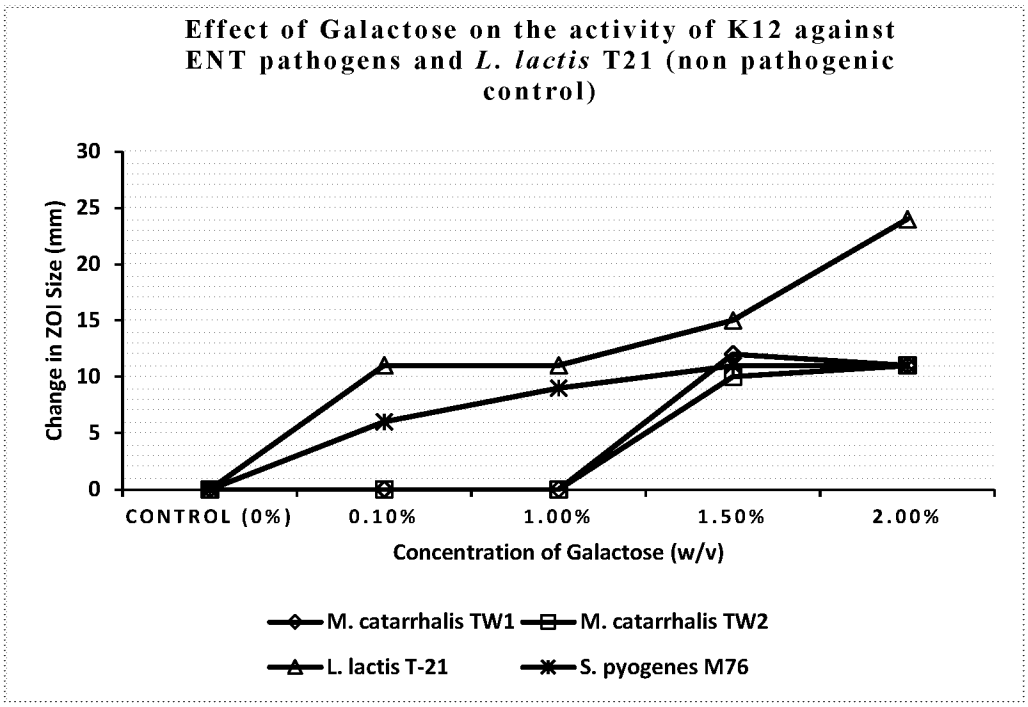


Figure 2

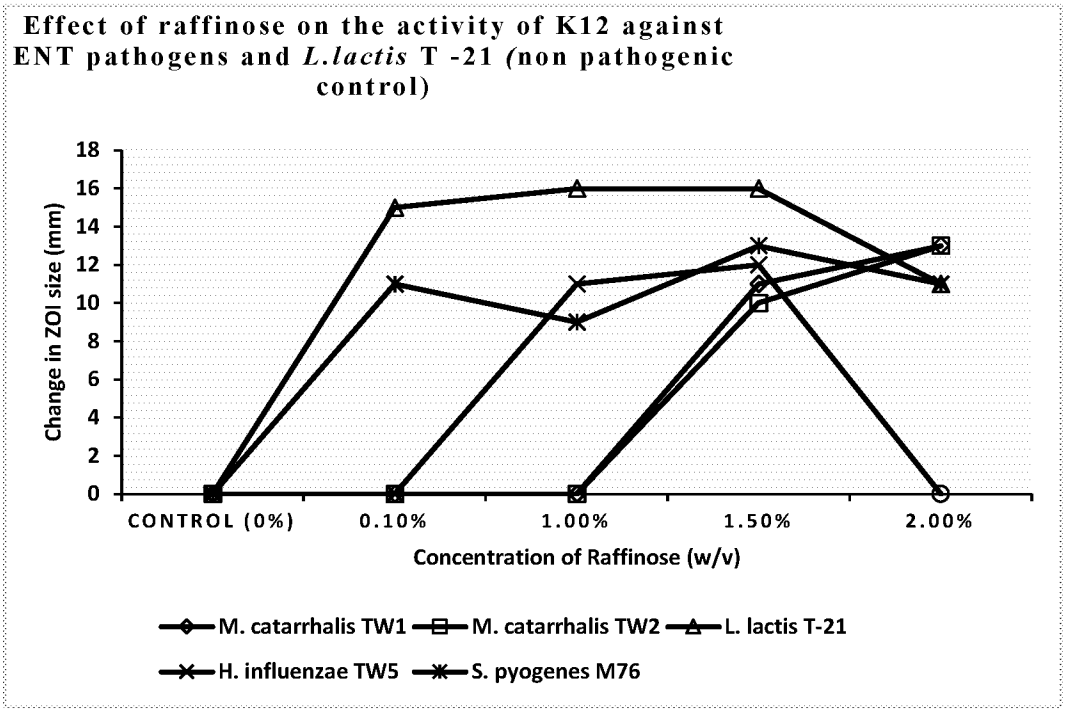


Figure 3

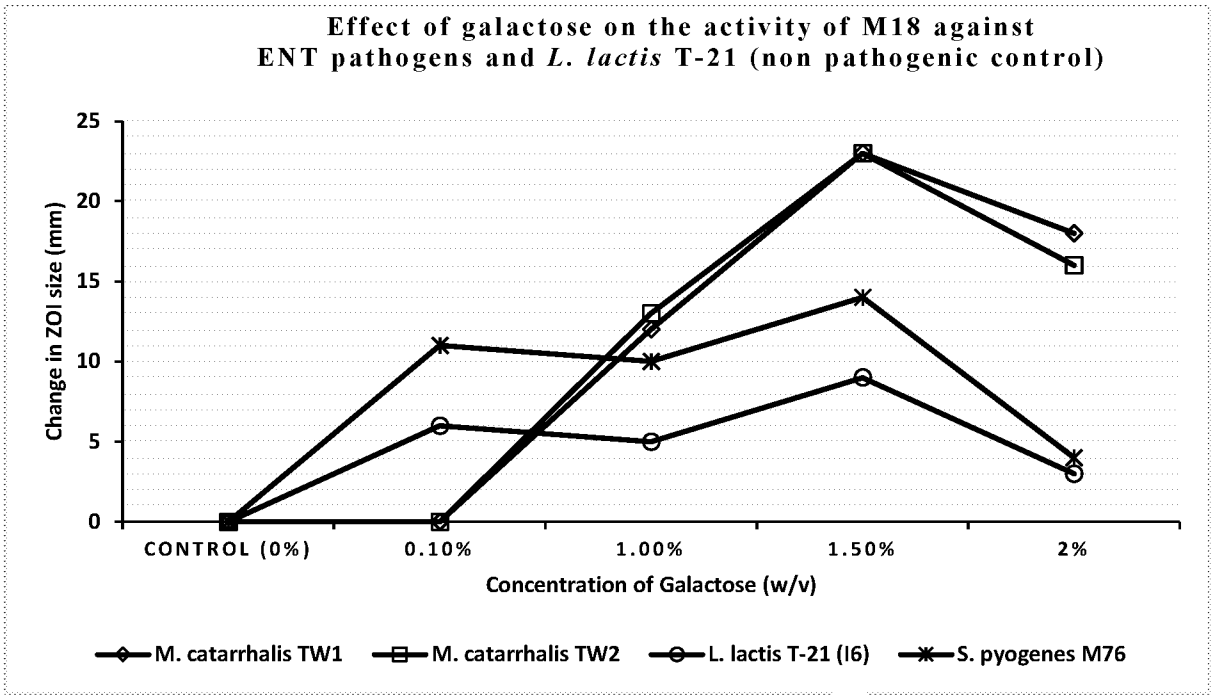


Figure 4

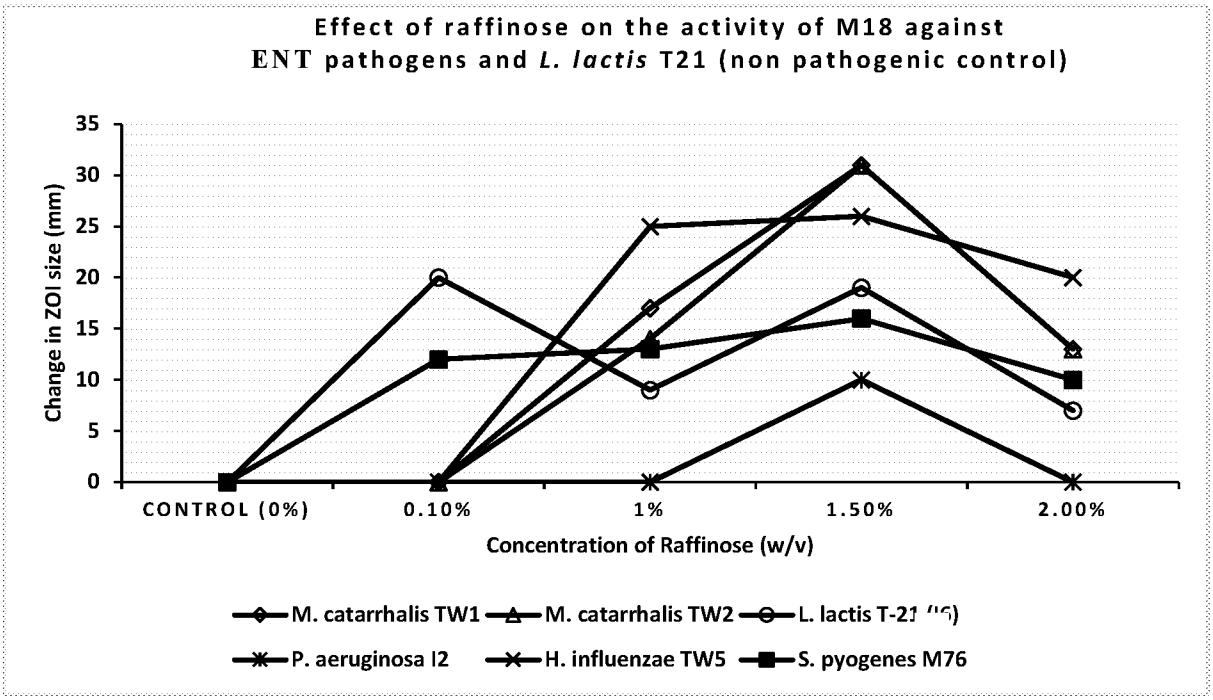
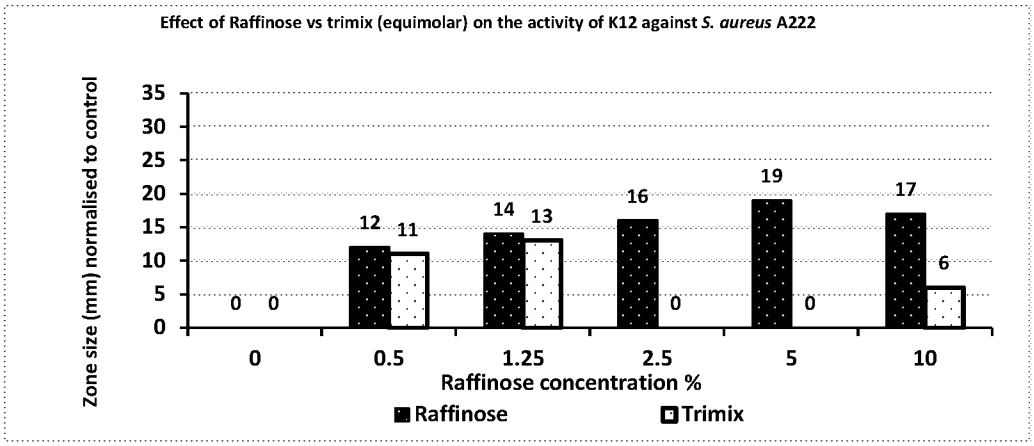
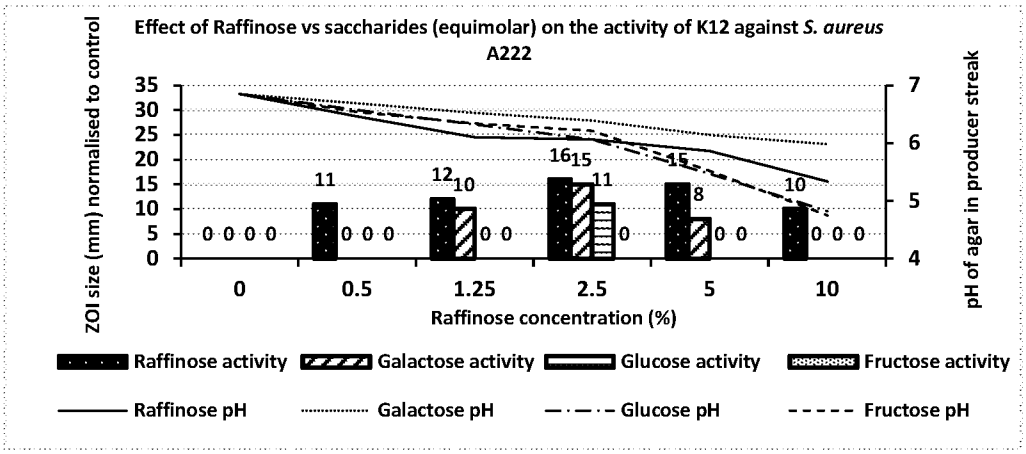


Figure 5

(a)


Effect of Raffinose vs trimix (equimolar) on the activity of K12 against S. aureus A222
Zone size (mm) normalised to control
35
30
25
20
15
10
5
0
0 0
12 11
14 13
16 0
19 0
17 6
0
0.5
1.25
2.5
5
10
Raffinose concentration %
Raffinose
Trimix

(b)


Effect of Raffinose vs saccharides (equimolar) on the activity of K12 against S. aureus A222
ZOI size (mm) normalised to control
pH of agar in producer streak
35
30
25
20
15
10
5
0
7
6
5
4
0 0 0 0
11 0 0 0
12 10 0 0
16 15 11 0
15 8 0 0
10 0 0 0
0
0.5
1.25
2.5
5
10
Raffinose concentration (%)
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

(c)

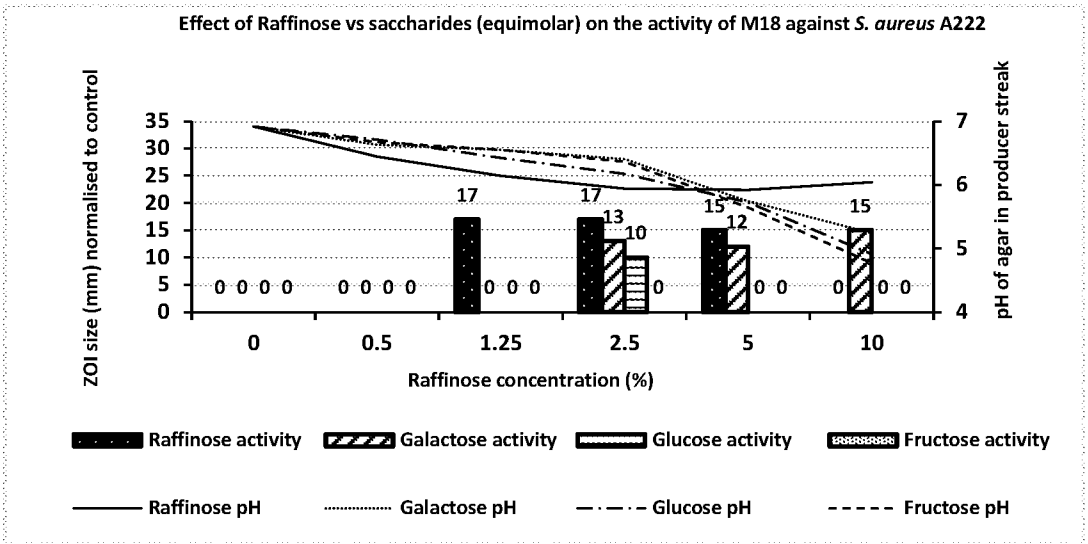
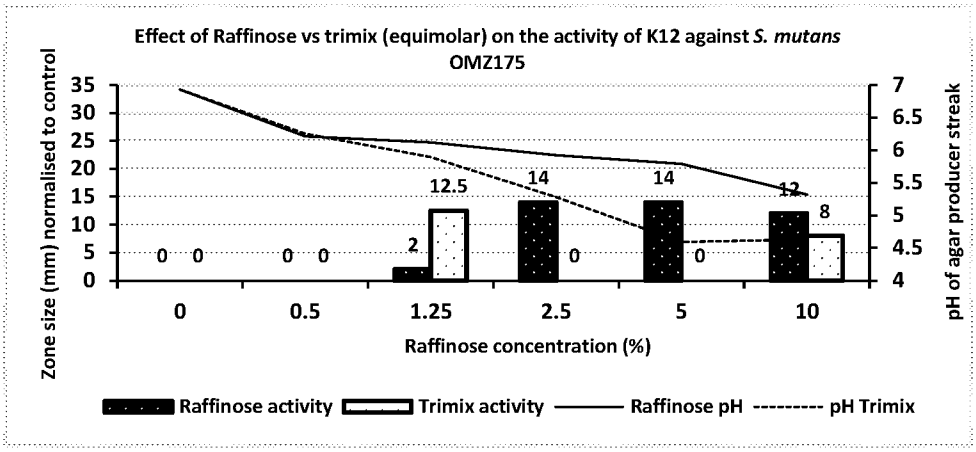
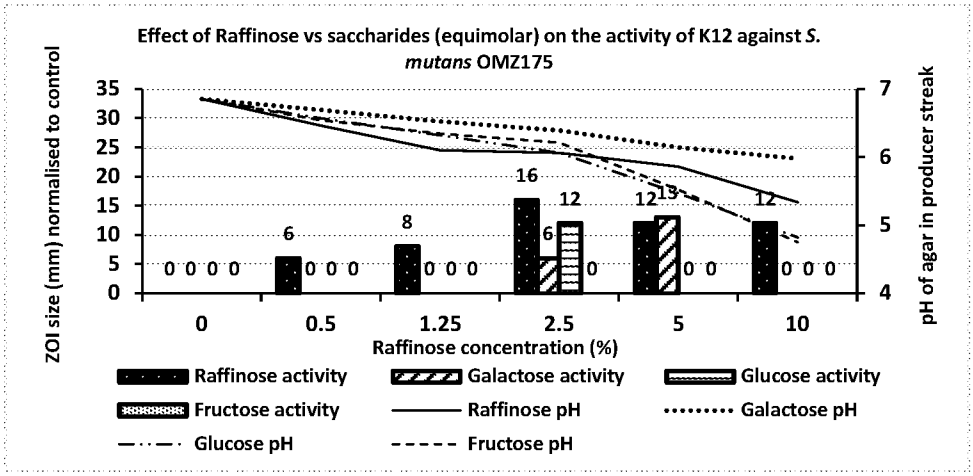

Effect of Raffinose vs saccharides (equimolar) on the activity of M18 against S. aureus A222
ZOI size (mm) normalised to control
pH of agar in producer streak
35
30
25
20
15
10
5
0
7
6
5
4
0 0 0 0
0 0 0 0
17 0 0 0
17 13 10 0
15 12 0 0
15 0 0 0
0
0.5
1.25
2.5
5
10
Raffinose concentration (%)
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

Figure 6

(a)


Effect of Raffinose vs trimix (equimolar) on the activity of K12 against S. mutans OMZ175
Zone size (mm) normalised to control
pH of agar producer streak
Raffinose concentration (%)
0
0.5
1.25
2.5
5
10
Raffinose activity
Trimix activity
Raffinose pH
pH Trimix

(b)


Effect of Raffinose vs saccharides (equimolar) on the activity of K12 against S. mutans OMZ175
ZOI size (mm) normalised to control
pH of agar in producer streak
Raffinose concentration (%)
0
0.5
1.25
2.5
5
10
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

(c)

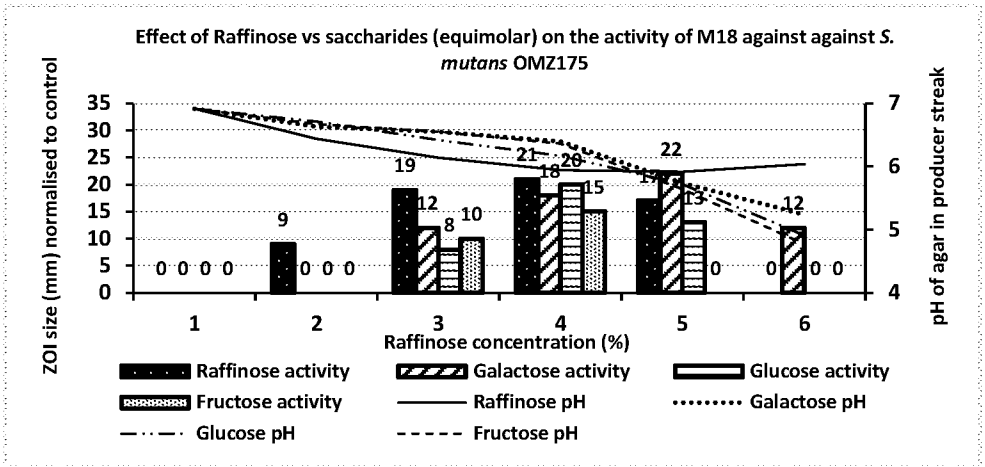
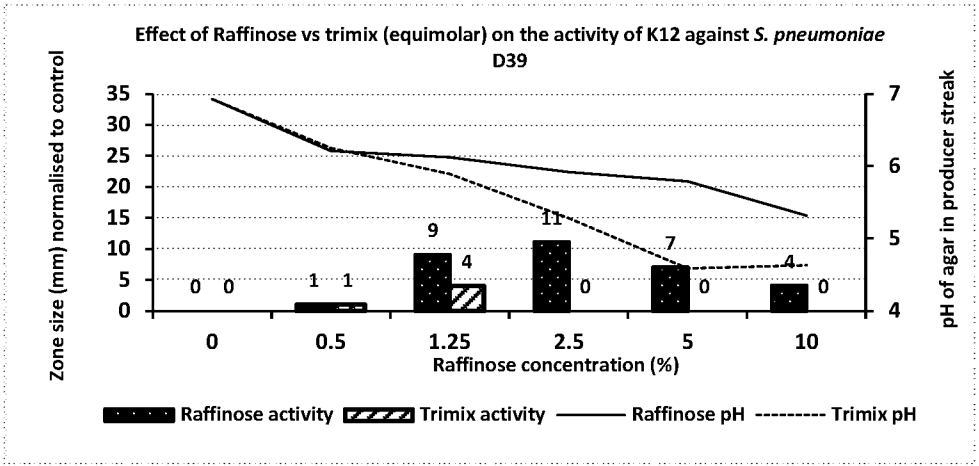
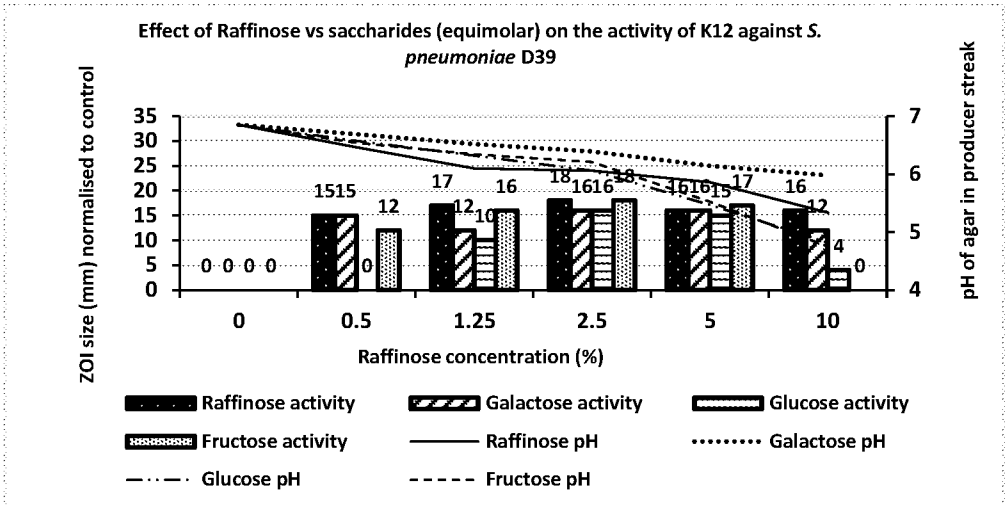

Effect of Raffinose vs saccharides (equimolar) on the activity of M18 against against S. mutans OMZ175
ZOI size (mm) normalised to control
pH of agar in producer streak
Raffinose concentration (%)
1
2
3
4
5
6
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

Figure 7

(a)


Effect of Raffinose vs trimix (equimolar) on the activity of K12 against S. pneumoniae D39
Zone size (mm) normalised to control
pH of agar in producer streak
Raffinose concentration (%)
0
0.5
1.25
2.5
5
10
Raffinose activity
Trimix activity
Raffinose pH
Trimix pH

(b)


Effect of Raffinose vs saccharides (equimolar) on the activity of K12 against S. pneumoniae D39
ZOI size (mm) normalised to control
pH of agar in producer streak
Raffinose concentration (%)
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

(c)

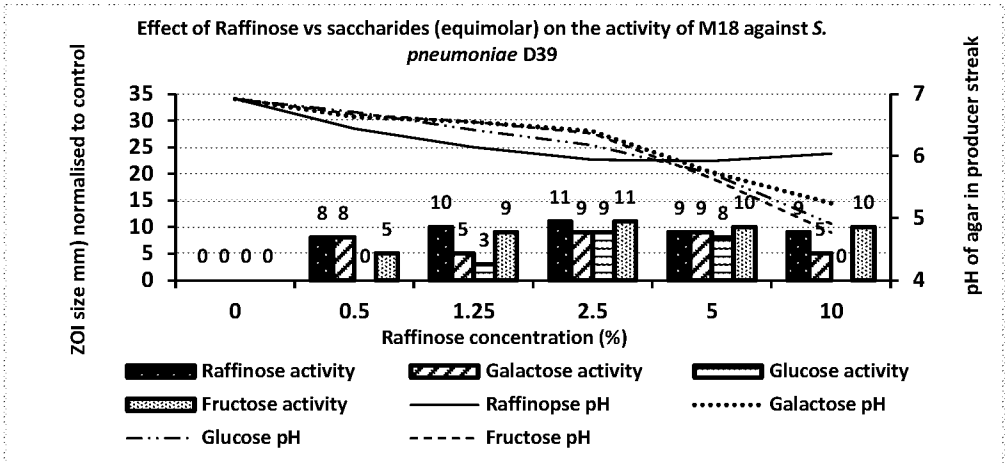
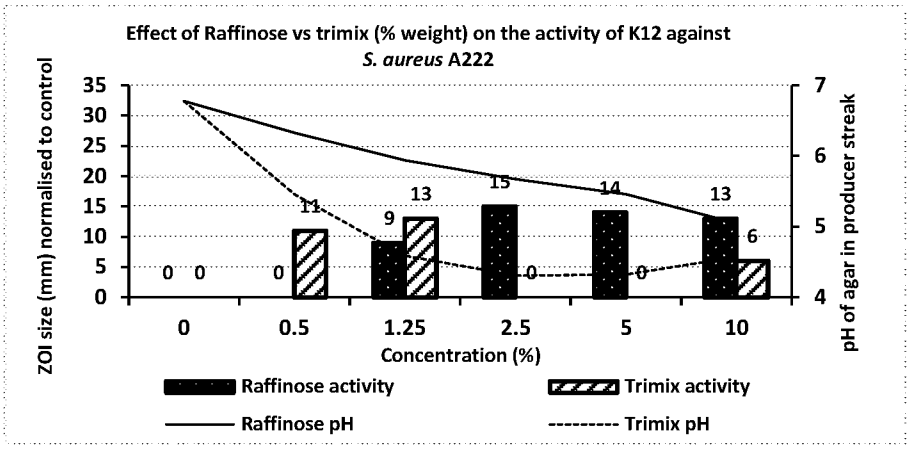
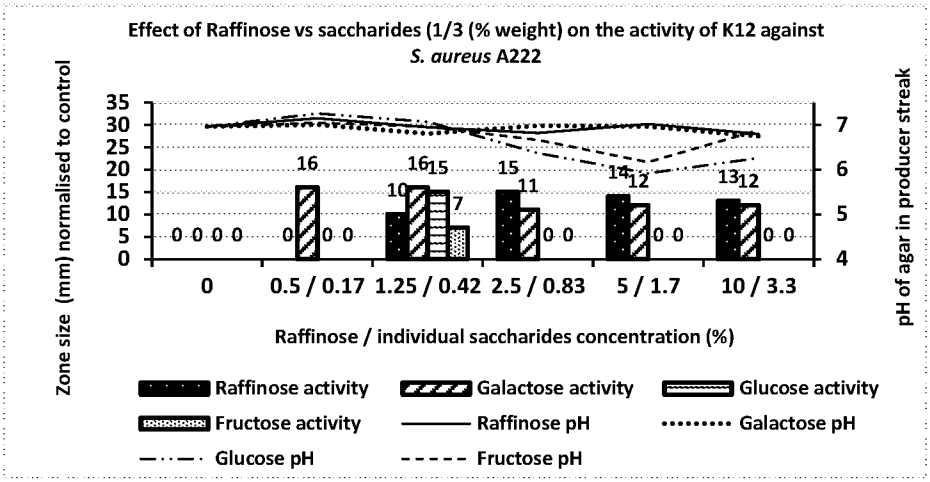

Effect of Raffinose vs saccharides (equimolar) on the activity of M18 against S. pneumoniae D39
ZOI size mm) normalised to control
pH of agar in producer streak
Raffinose concentration (%)
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinopse pH
Galactose pH
Glucose pH
Fructose pH

Figure 8

(a)


Effect of Raffinose vs trimix (% weight) on the activity of K12 against S. aureus A222
ZOI size (mm) normalised to control
pH of agar in producer streak
Concentration (%)
0
0.5
1.25
2.5
5
10
Raffinose activity
Trimix activity
Raffinose pH
Trimix pH

(b)


Effect of Raffinose vs saccharides (1/3 (% weight) on the activity of K12 against S. aureus A222
Zone size (mm) normalised to control
pH of agar in producer streak
Raffinose / individual saccharides concentration (%)
0
0.5 / 0.17
1.25 / 0.42
2.5 / 0.83
5 / 1.7
10 / 3.3
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

(c)

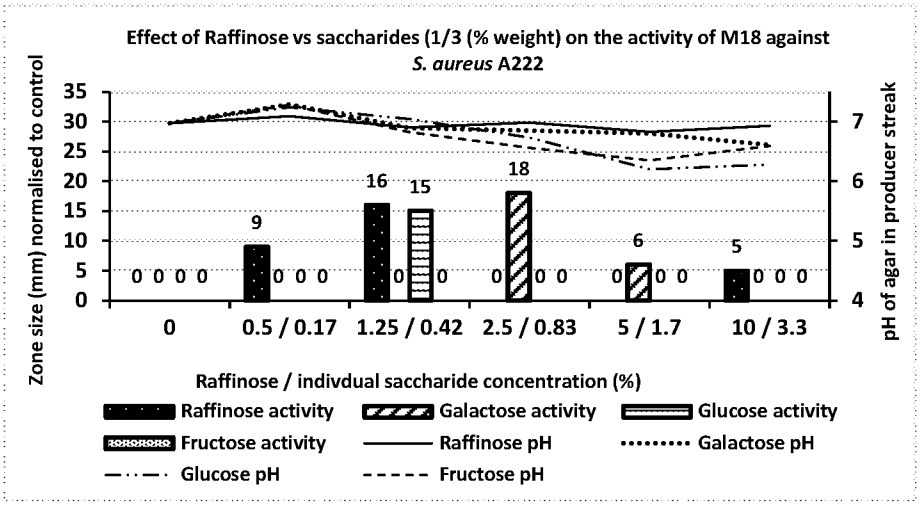
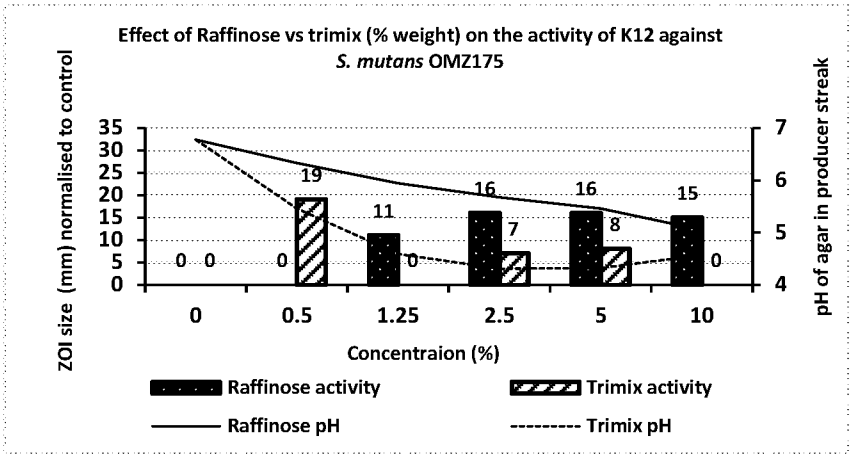
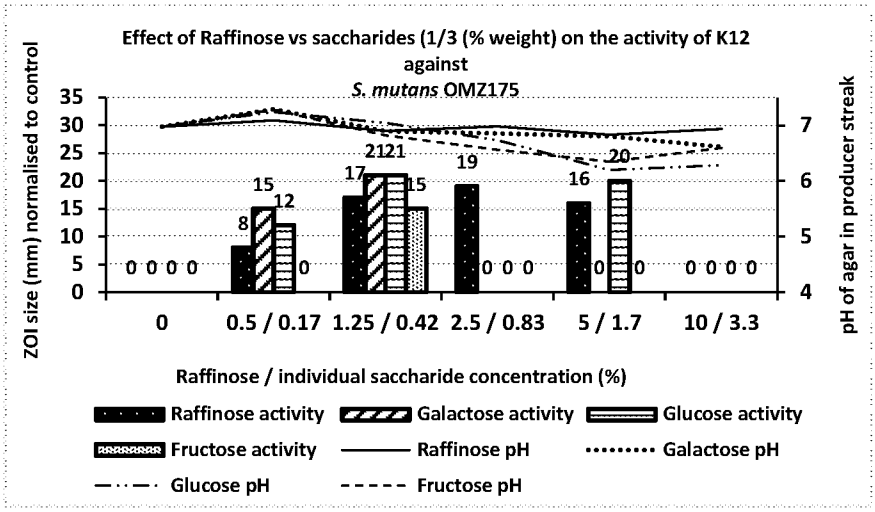

Effect of Raffinose vs saccharides (1/3 (% weight) on the activity of M18 against S. aureus A222
Zone size (mm) normalised to control
pH of agar in producer streak
Raffinose / indivdual saccharide concentration (%)
0
0.5 / 0.17
1.25 / 0.42
2.5 / 0.83
5 / 1.7
10 / 3.3
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

Figure 9

(a)


Effect of Raffinose vs trimix (% weight) on the activity of K12 against S. mutans OMZ175
ZOI size (mm) normalised to control
pH of agar in producer streak
Concentraion (%)
0
0.5
1.25
2.5
5
10
Raffinose activity
Trimix activity
Raffinose pH
Trimix pH

(b)


Effect of Raffinose vs saccharides (1/3 (% weight) on the activity of K12 against S. mutans OMZ175
ZOI size (mm) normalised to control
pH of agar in producer streak
Raffinose / individual saccharide concentration (%)
0
0.5 / 0.17
1.25 / 0.42
2.5 / 0.83
5 / 1.7
10 / 3.3
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH
Glucose pH
Fructose pH

(c)

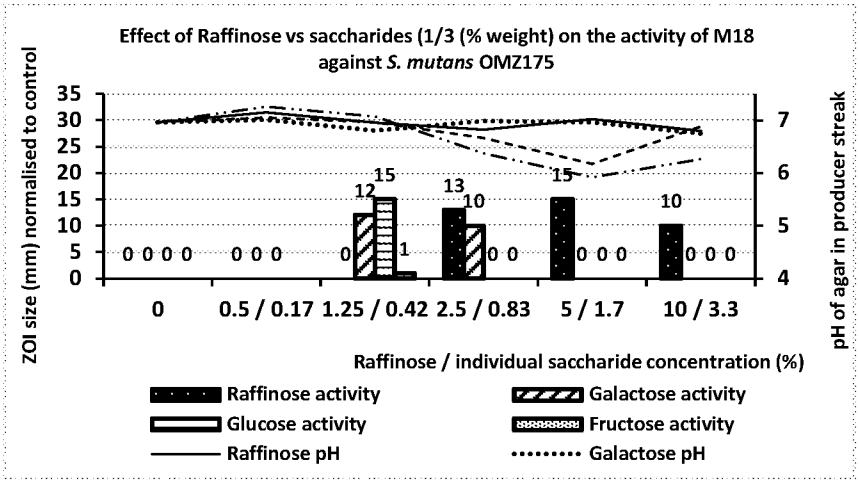
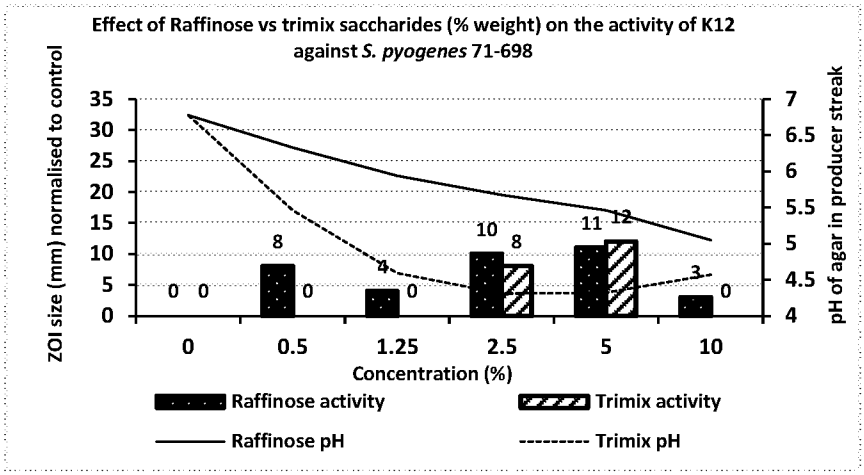
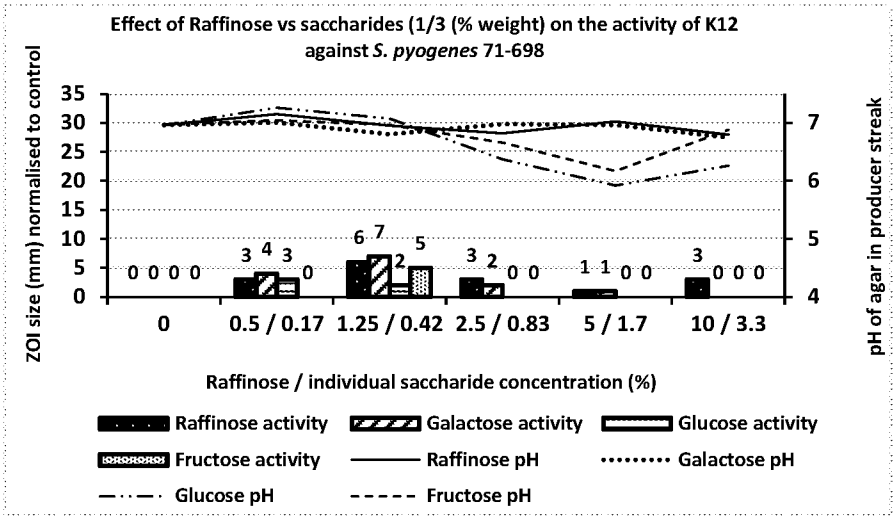

Effect of Raffinose vs saccharides (1/3 (% weight) on the activity of M18 against S. mutans OMZ175
ZOI size (mm) normalised to control
pH of agar in producer streak
Raffinose / individual saccharide concentration (%)
0
0.5 / 0.17
1.25 / 0.42
2.5 / 0.83
5 / 1.7
10 / 3.3
Raffinose activity
Galactose activity
Glucose activity
Fructose activity
Raffinose pH
Galactose pH

Figure 10

(a)



(b)



(c)

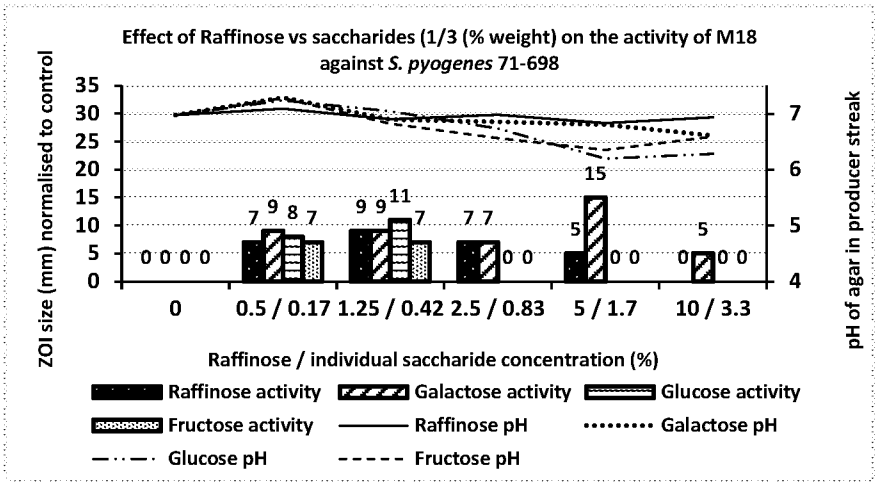
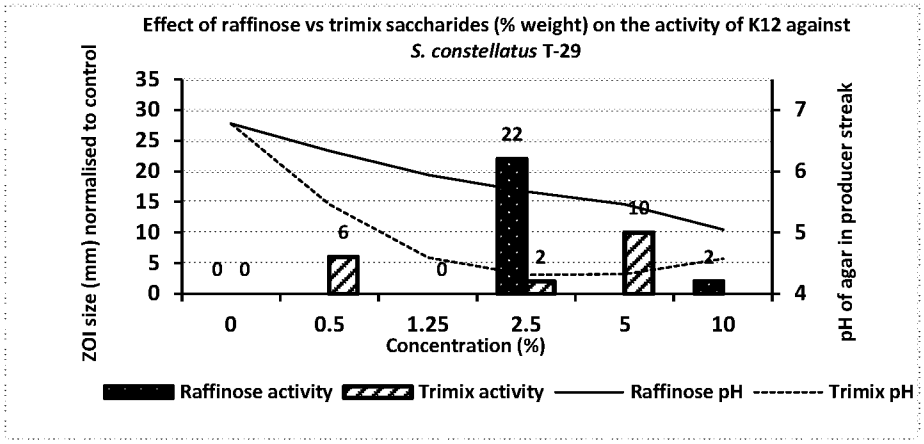
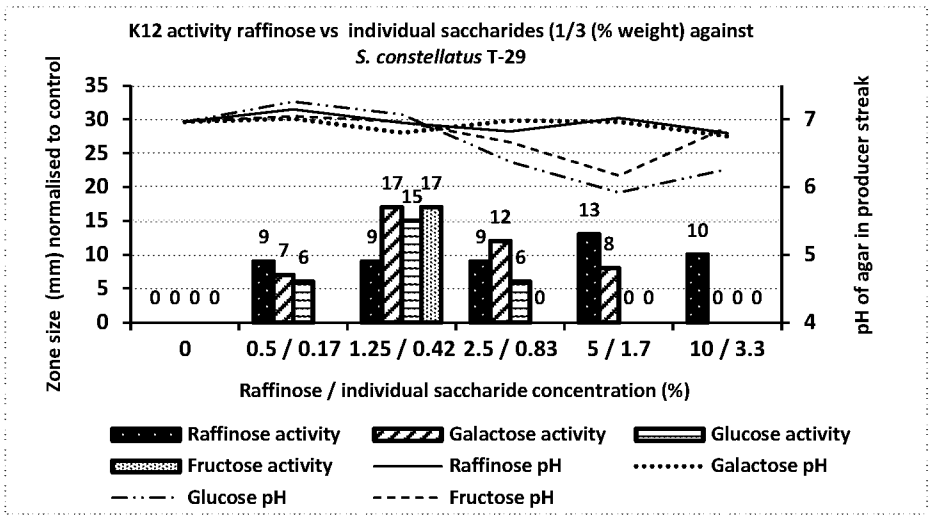


Figure 11

(a)



(b)



(c)

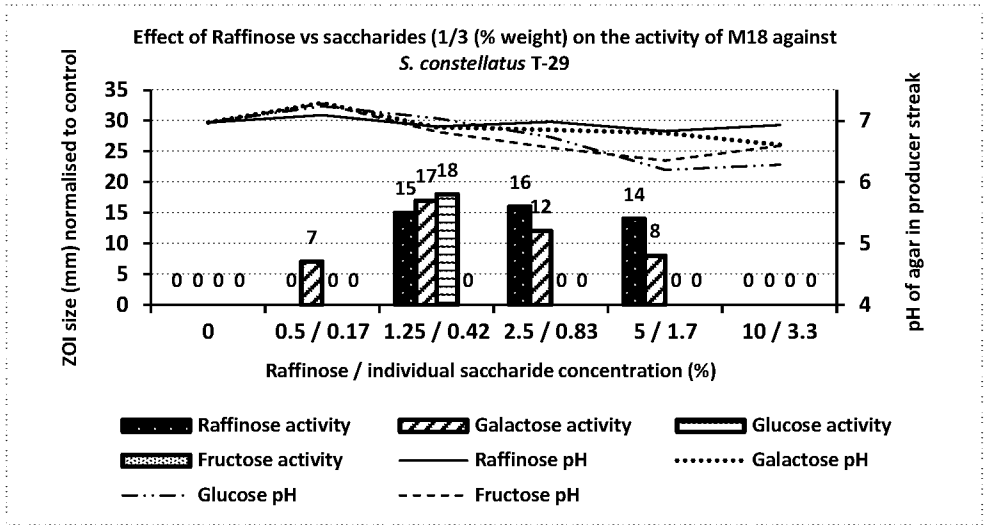
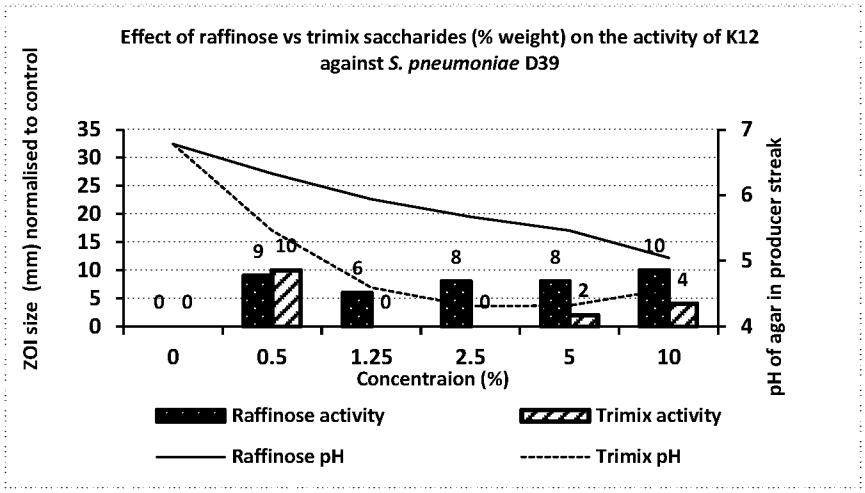
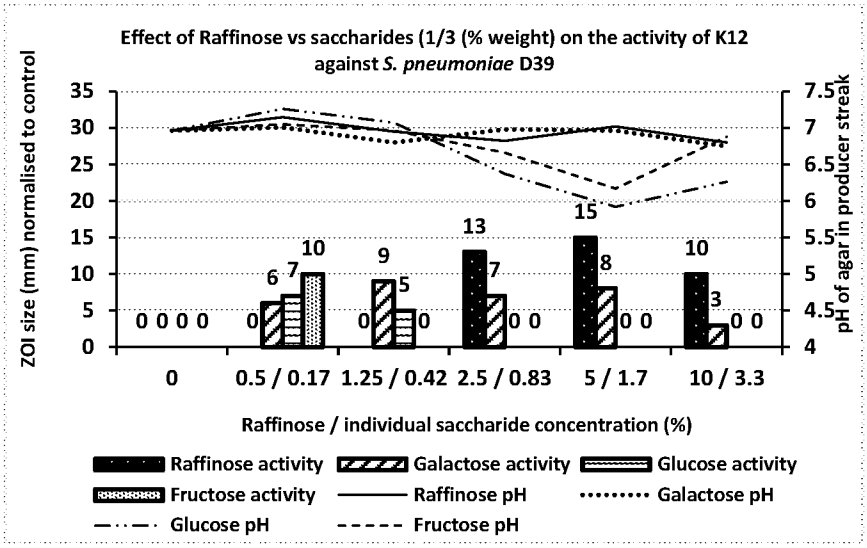


Figure 12

(a)



(b)



(c)

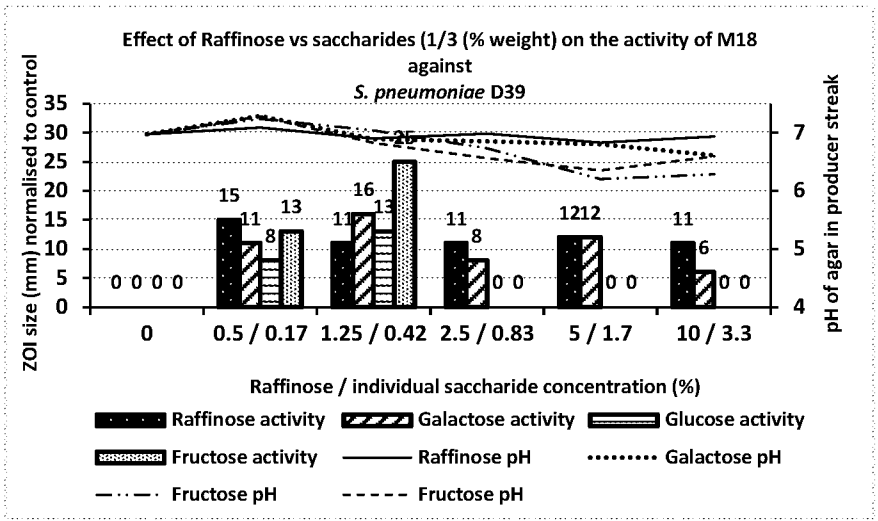


Figure 13 (a)

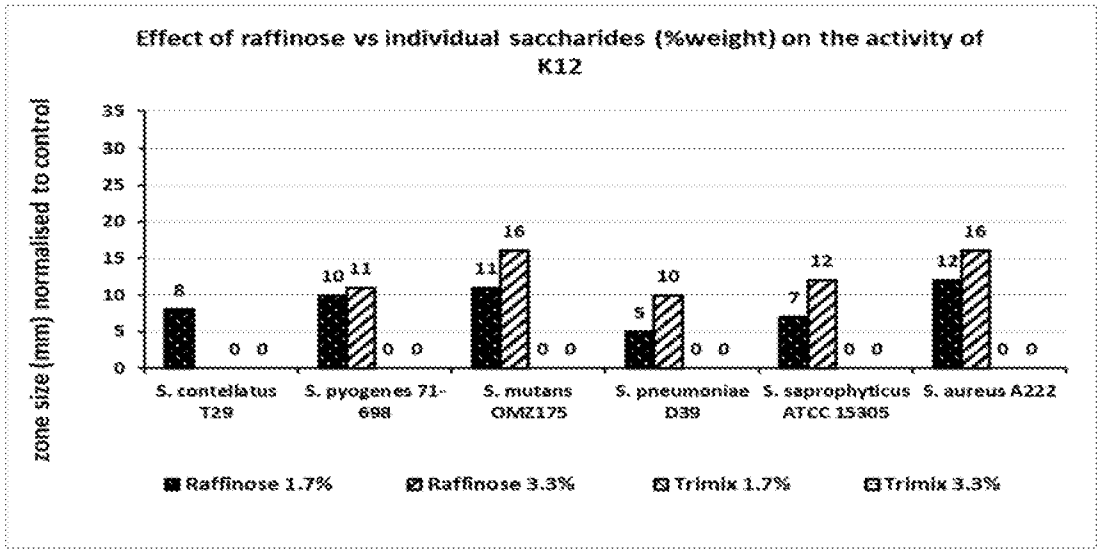


Figure 13 (b)

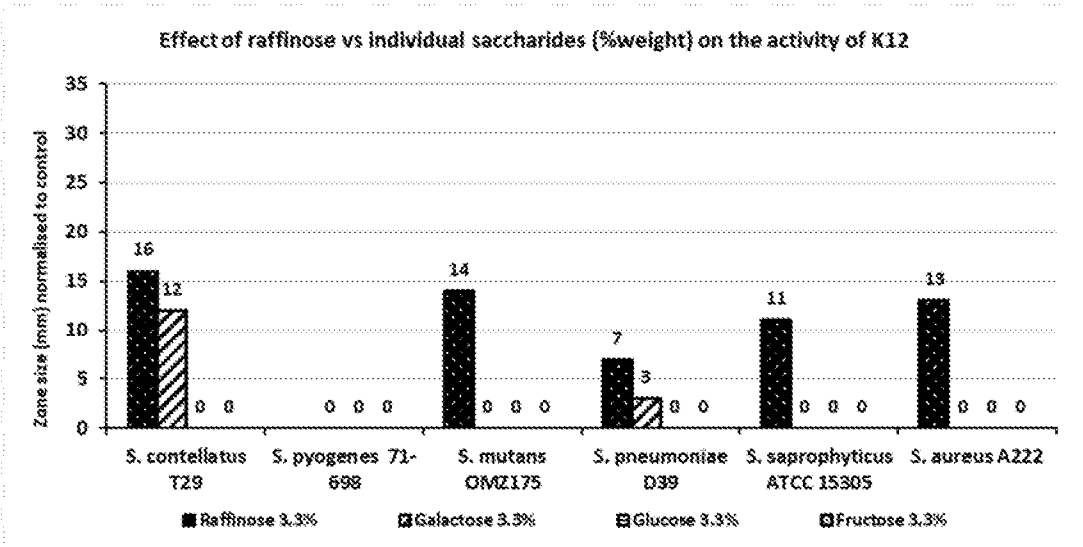


Figure 13 (c)

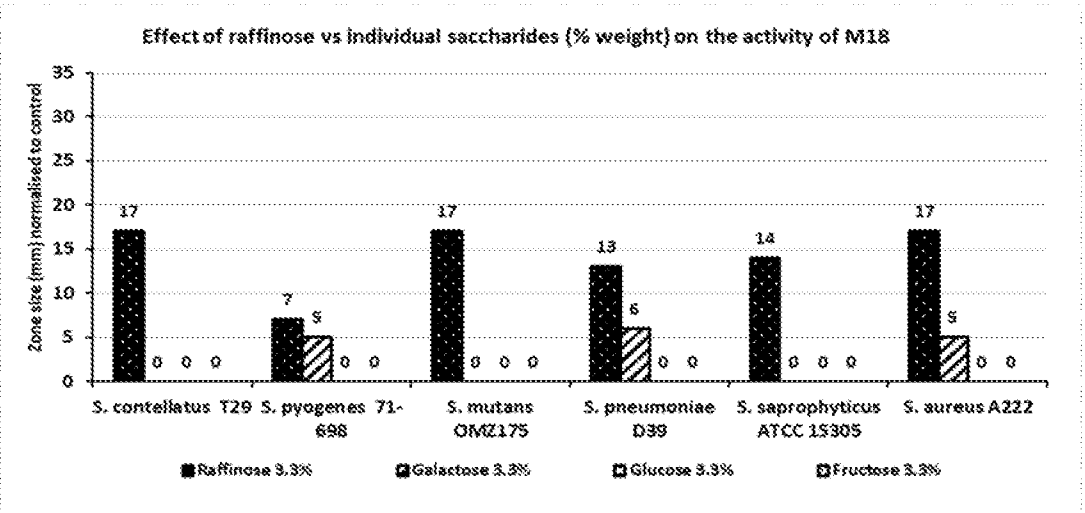


Figure 14

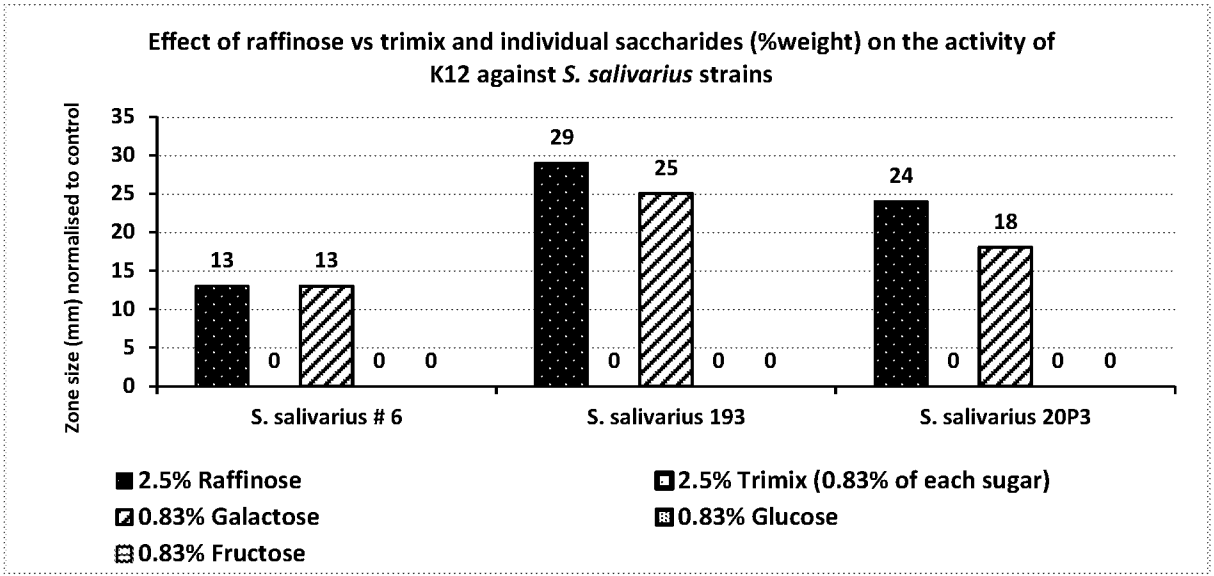


Figure 15

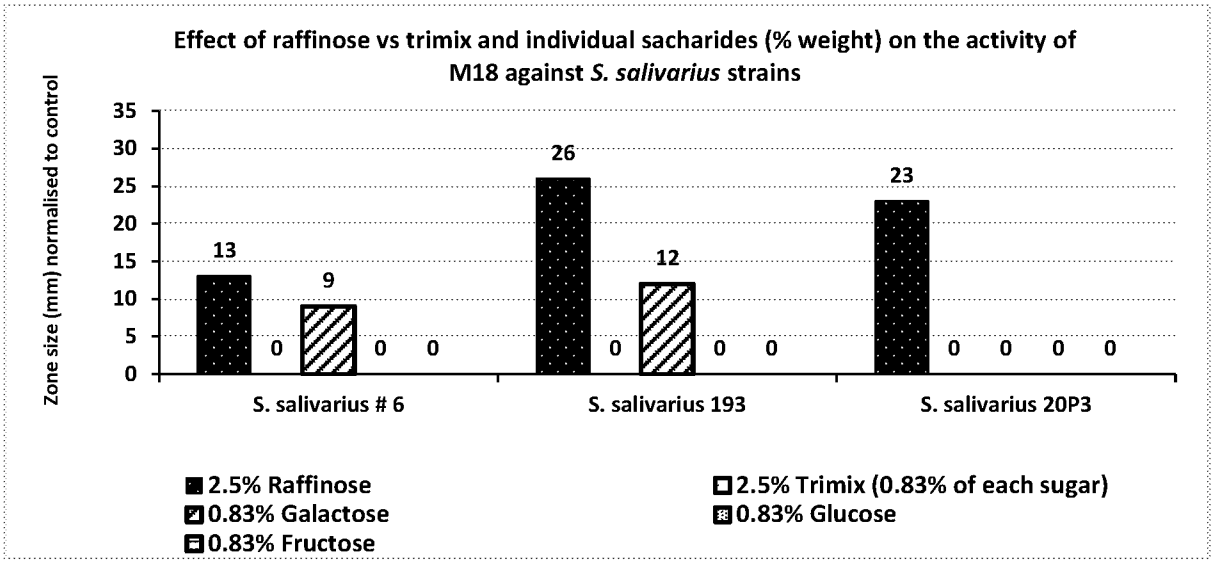


Figure 16

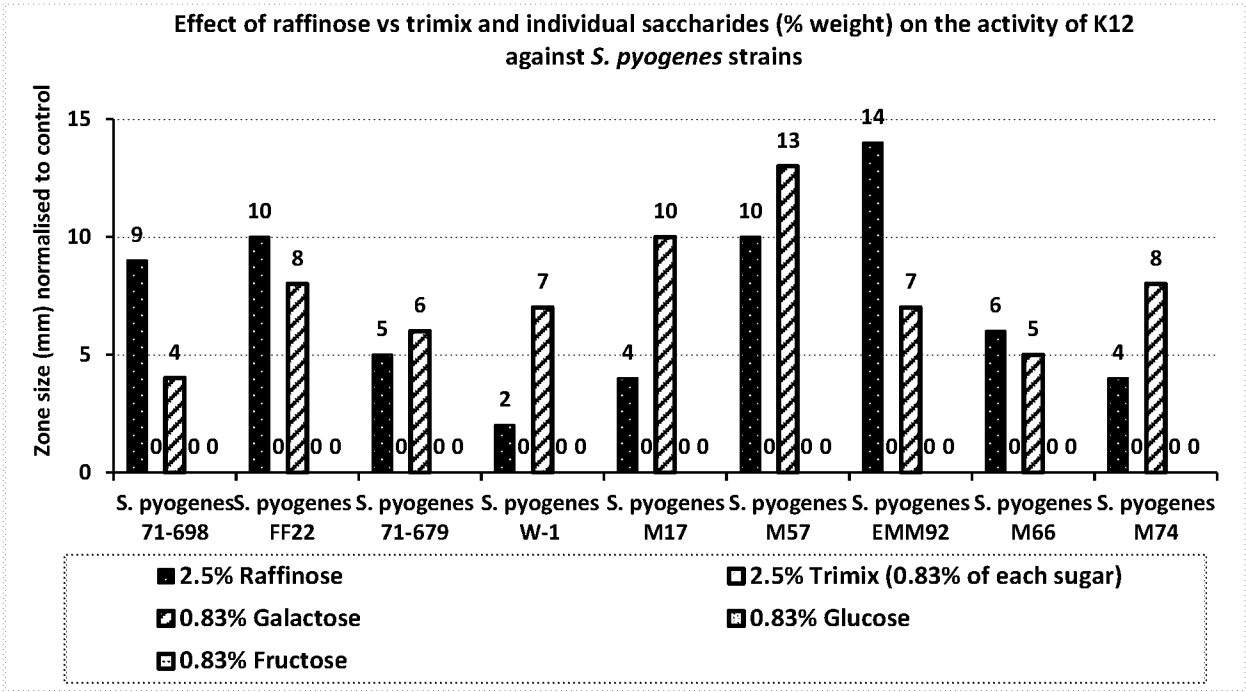


Figure 17

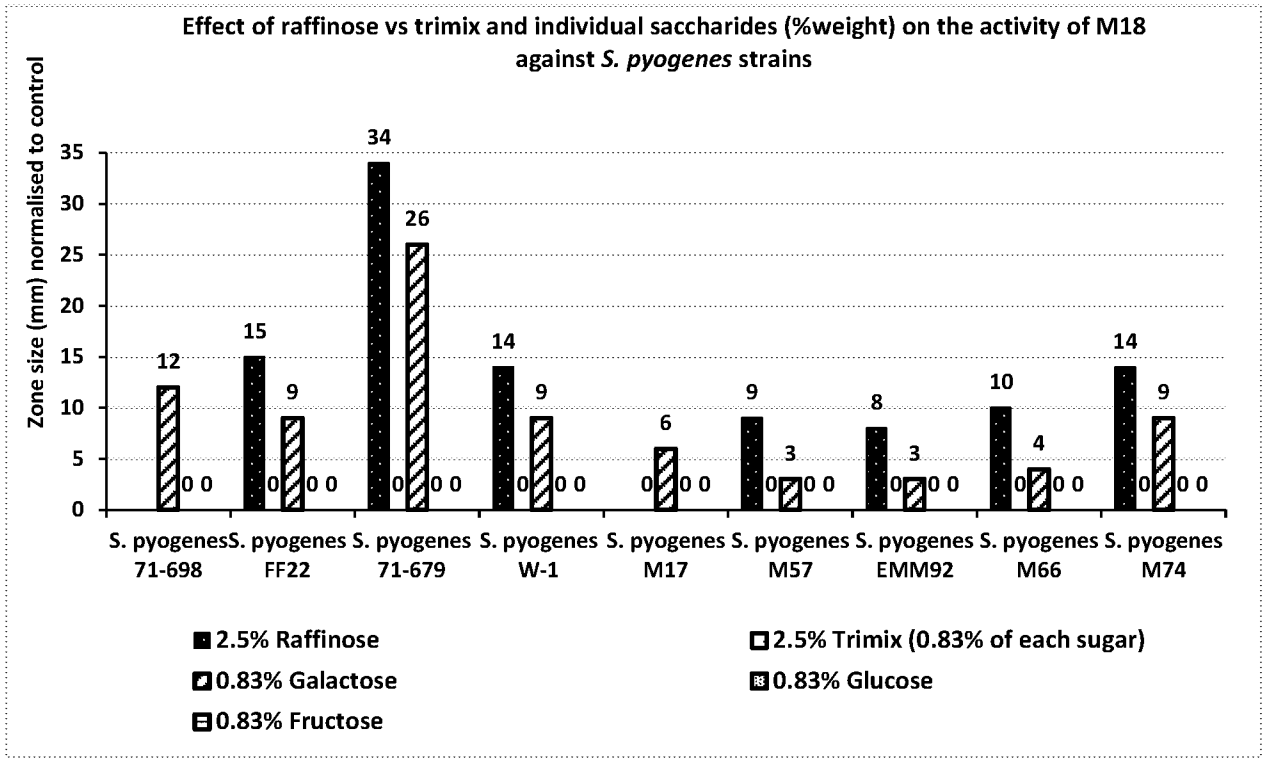


Figure 18

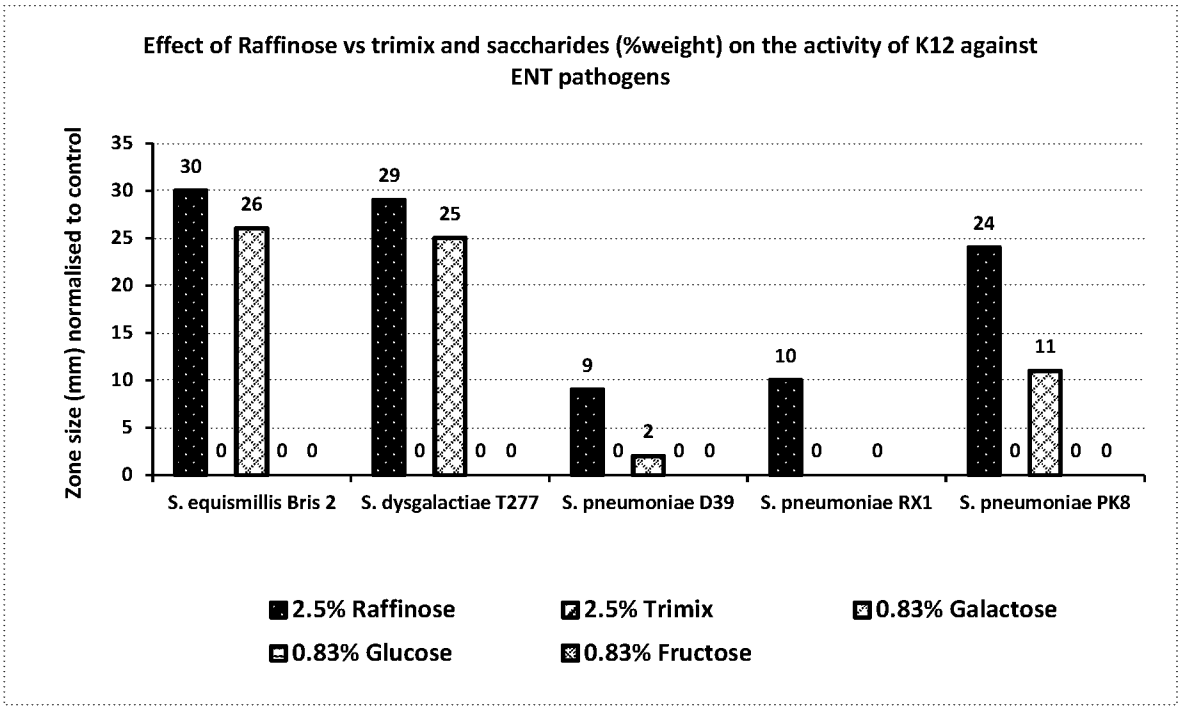


Figure 19

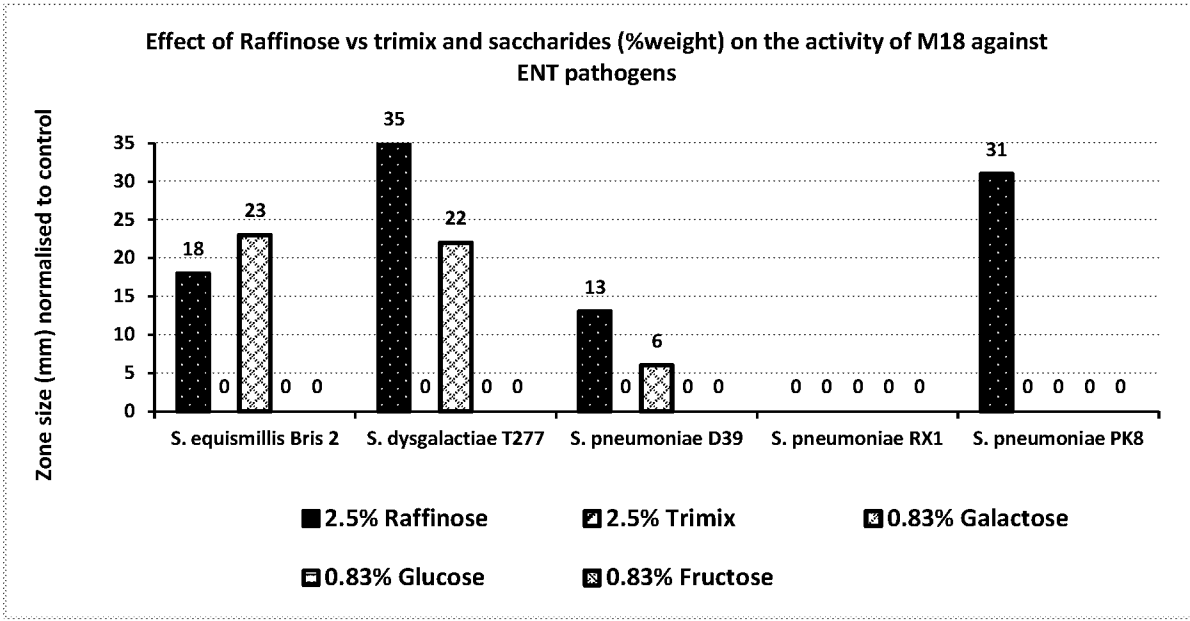


Figure 20

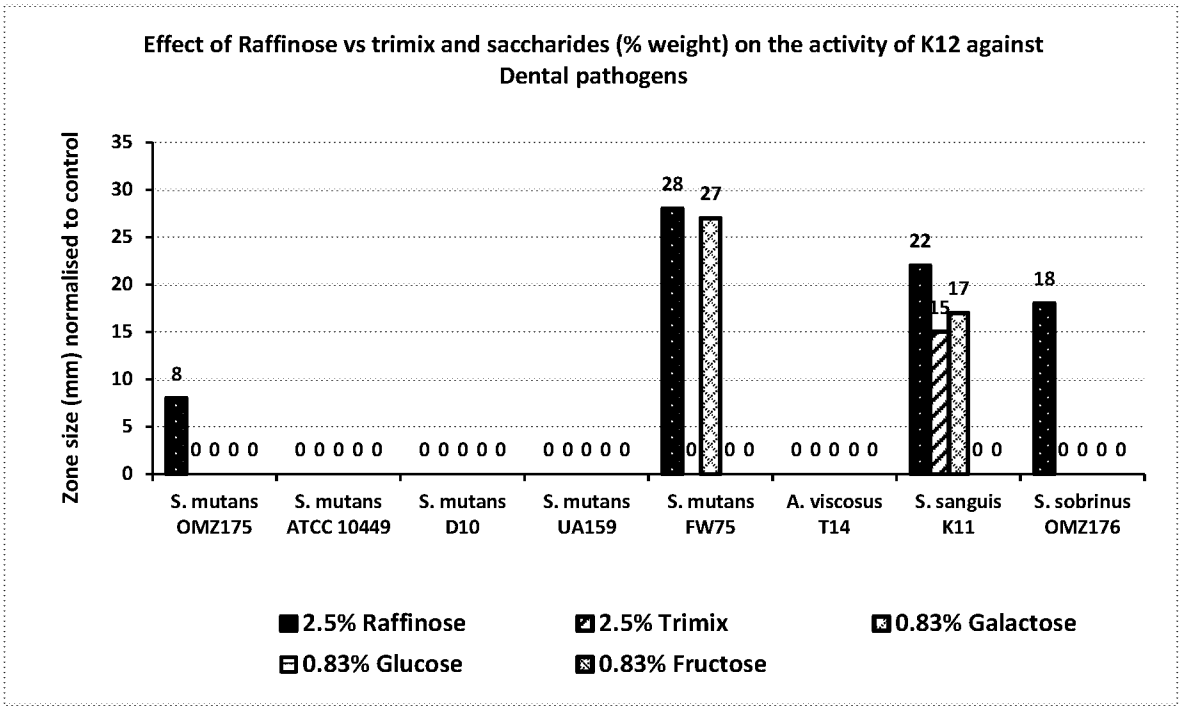


Figure 21

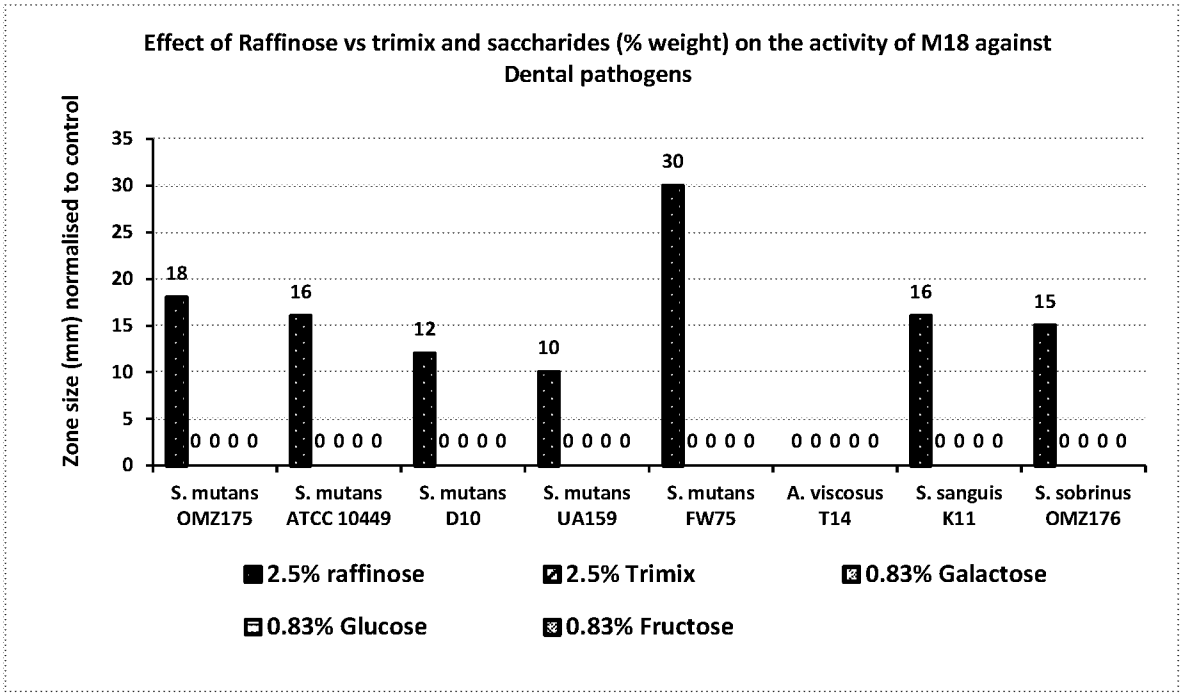


Figure 22

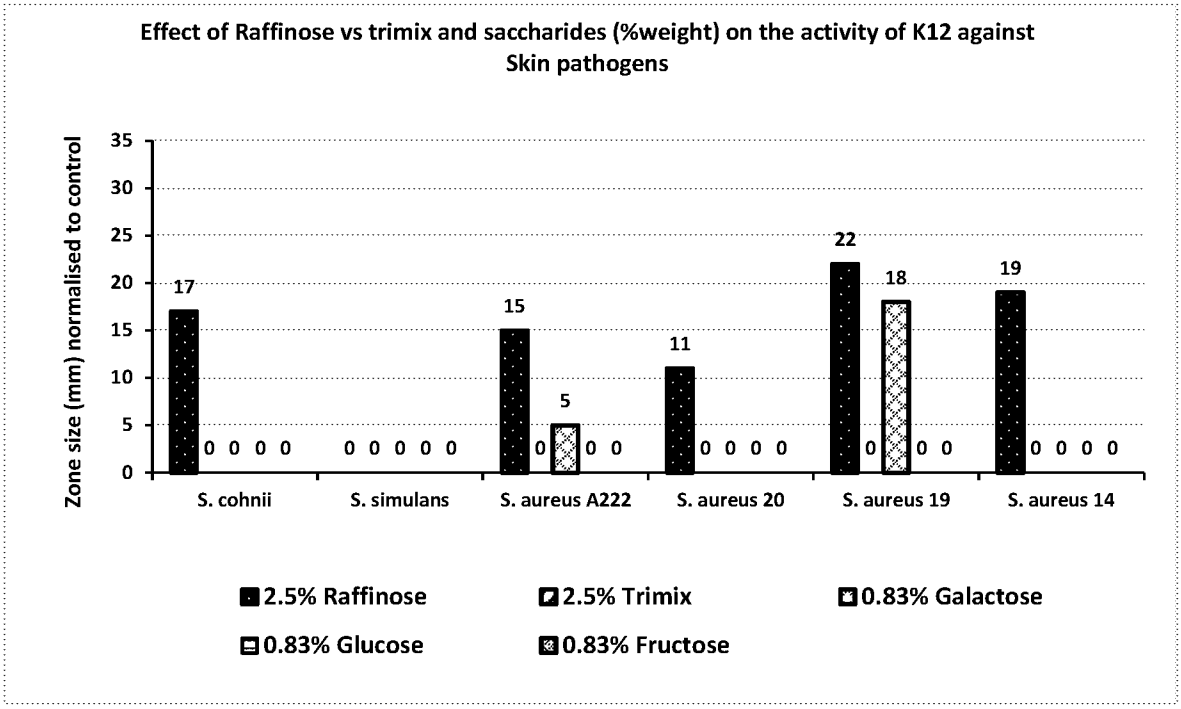


Figure 23

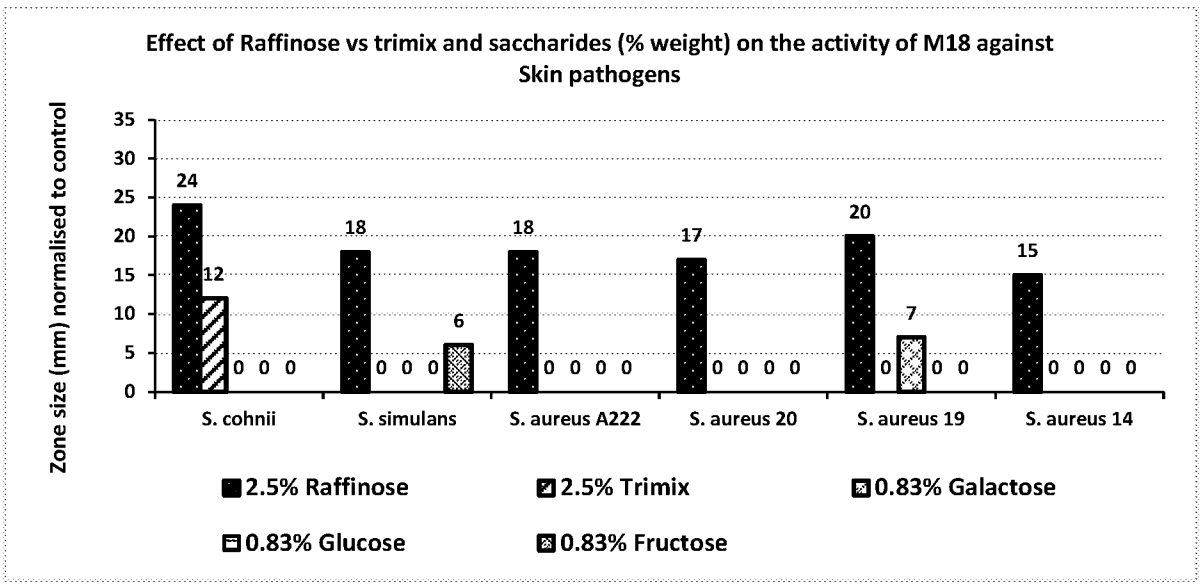


Figure 24

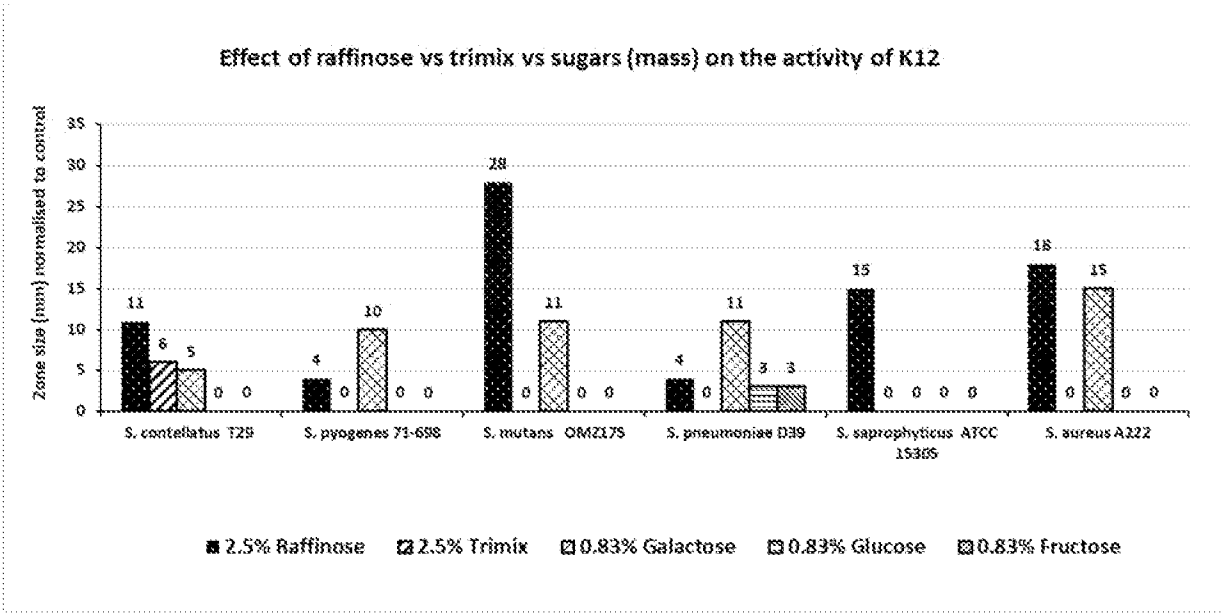


Figure 25

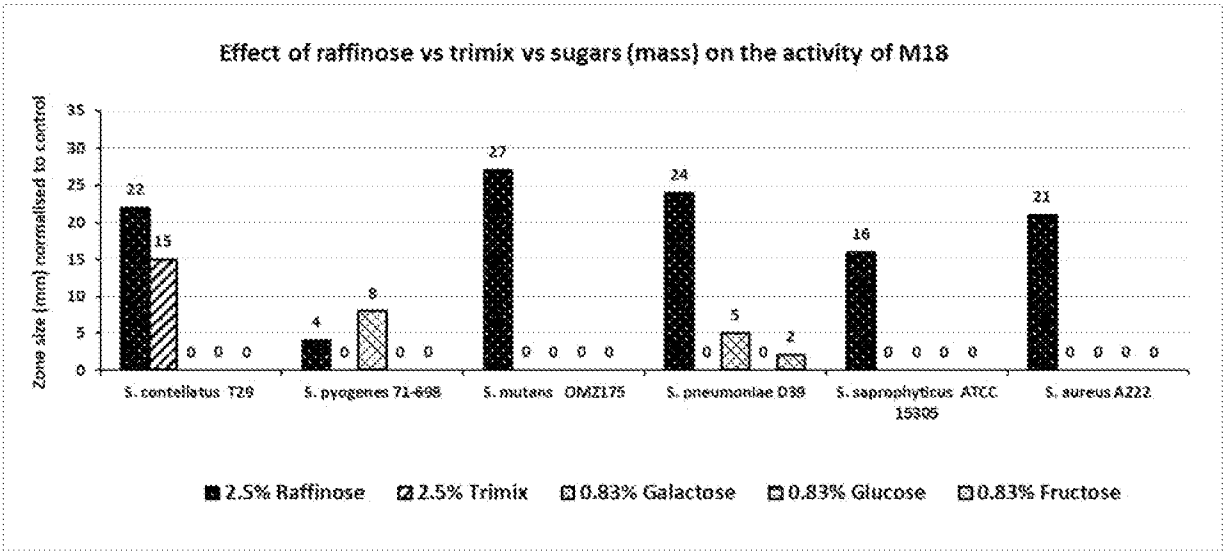


Figure 26

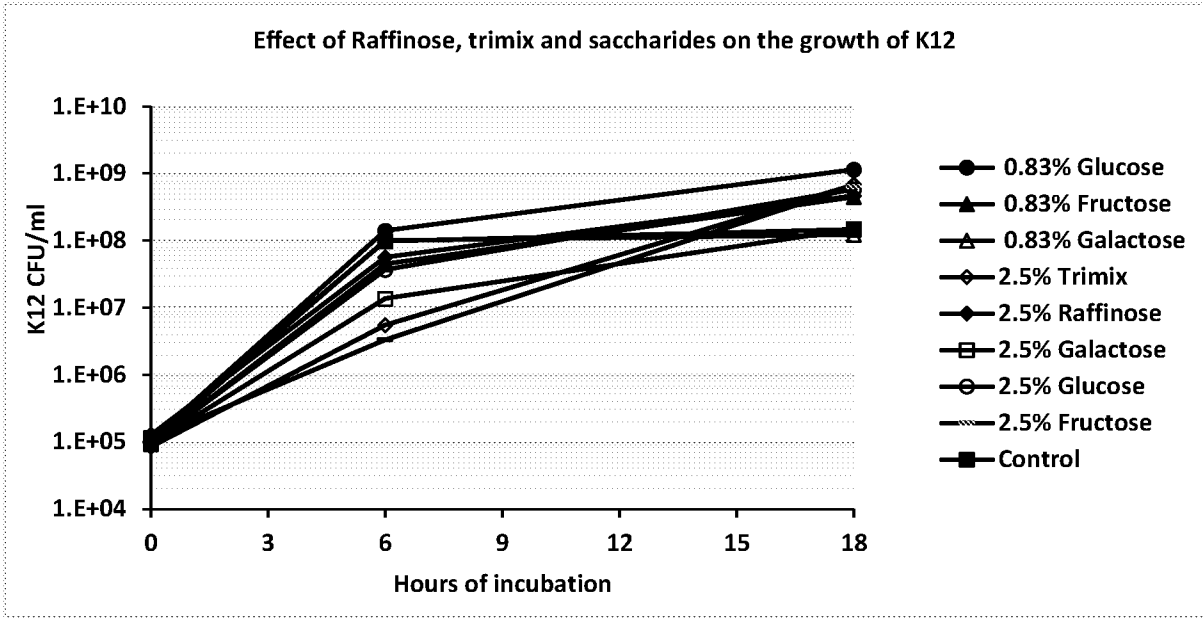


Figure 27

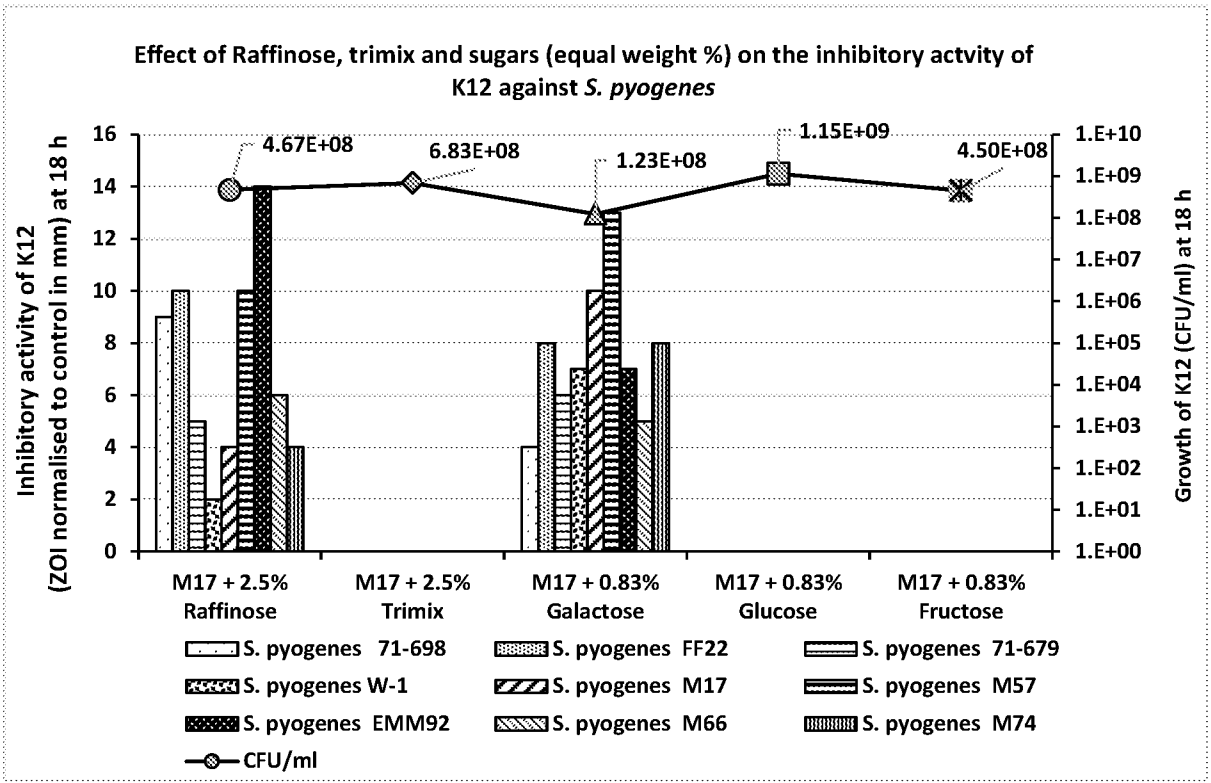


Figure 28

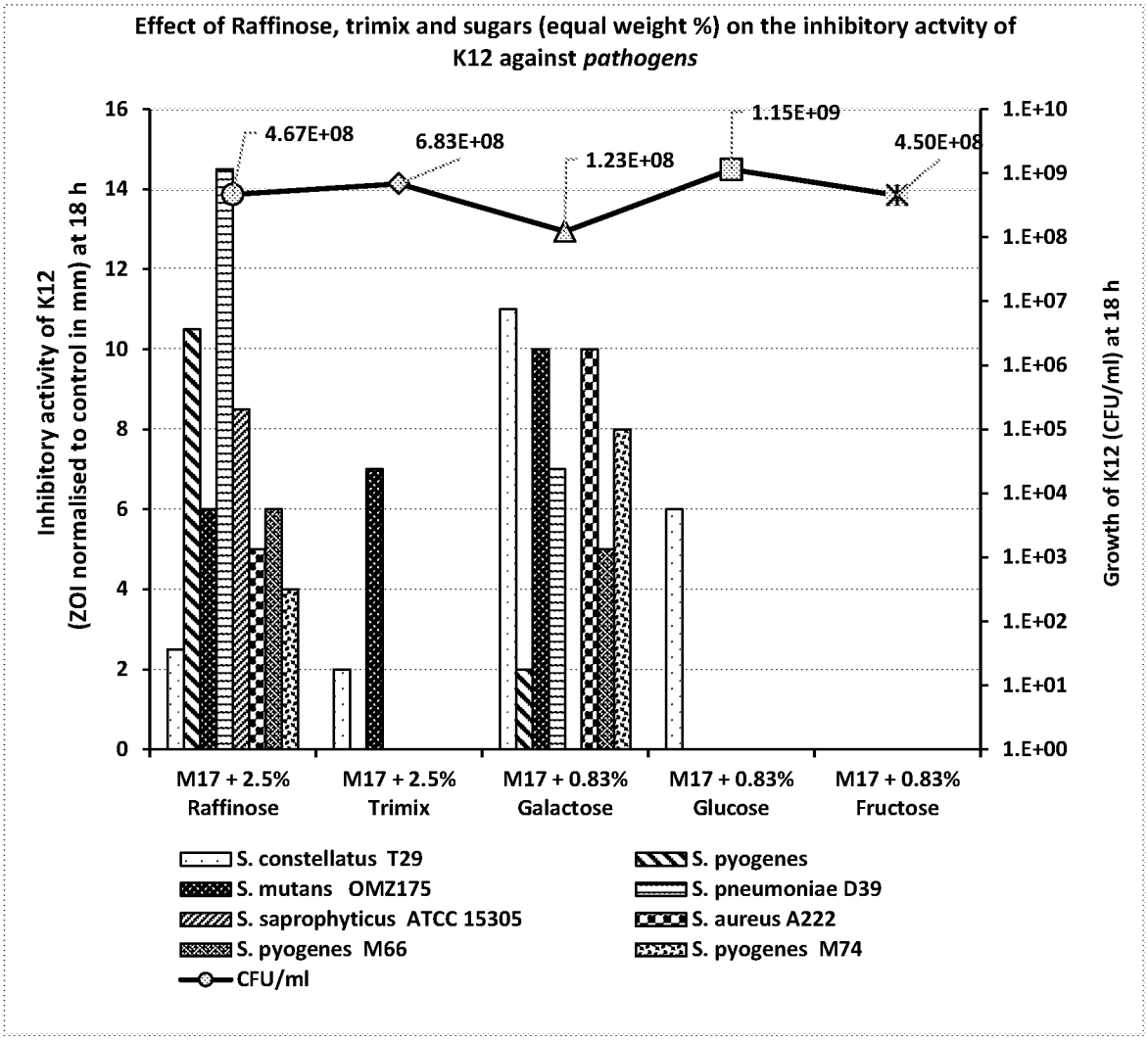

Effect of Raffinose, trimix and sugars (equal weight %) on the inhibitory actvity of K12 against *pathogens*
Inhibitory activity of K12 (ZOI normalised to control in mm) at 18 h
Growth of K12 (CFU/ml) at 18 h
4.67E+08
6.83E+08
1.23E+08
1.15E+09
4.50E+08
M17 + 2.5% Raffinose
M17 + 2.5% Trimix
M17 + 0.83% Galactose
M17 + 0.83% Glucose
M17 + 0.83% Fructose
S. constellatus T29
S. mutans OMZ175
S. saprophyticus ATCC 15305
S. pyogenes M66
CFU/ml
S. pyogenes
S. pneumoniae D39
S. aureus A222
S. pyogenes M74

Figure 28A

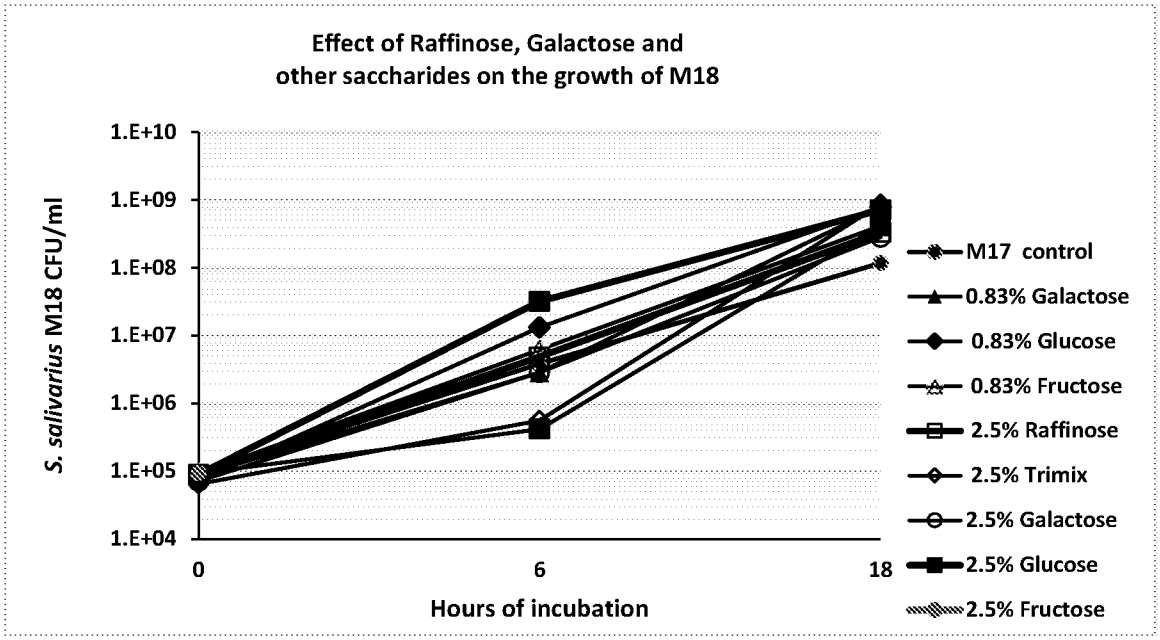

Effect of Raffinose, Galactose and other saccharides on the growth of M18
S. salivarius M18 CFU/ml
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
0
6
18
Hours of incubation
M17 control
0.83% Galactose
0.83% Glucose
0.83% Fructose
2.5% Raffinose
2.5% Trimix
2.5% Galactose
2.5% Glucose
2.5% Fructose

Figure 28B

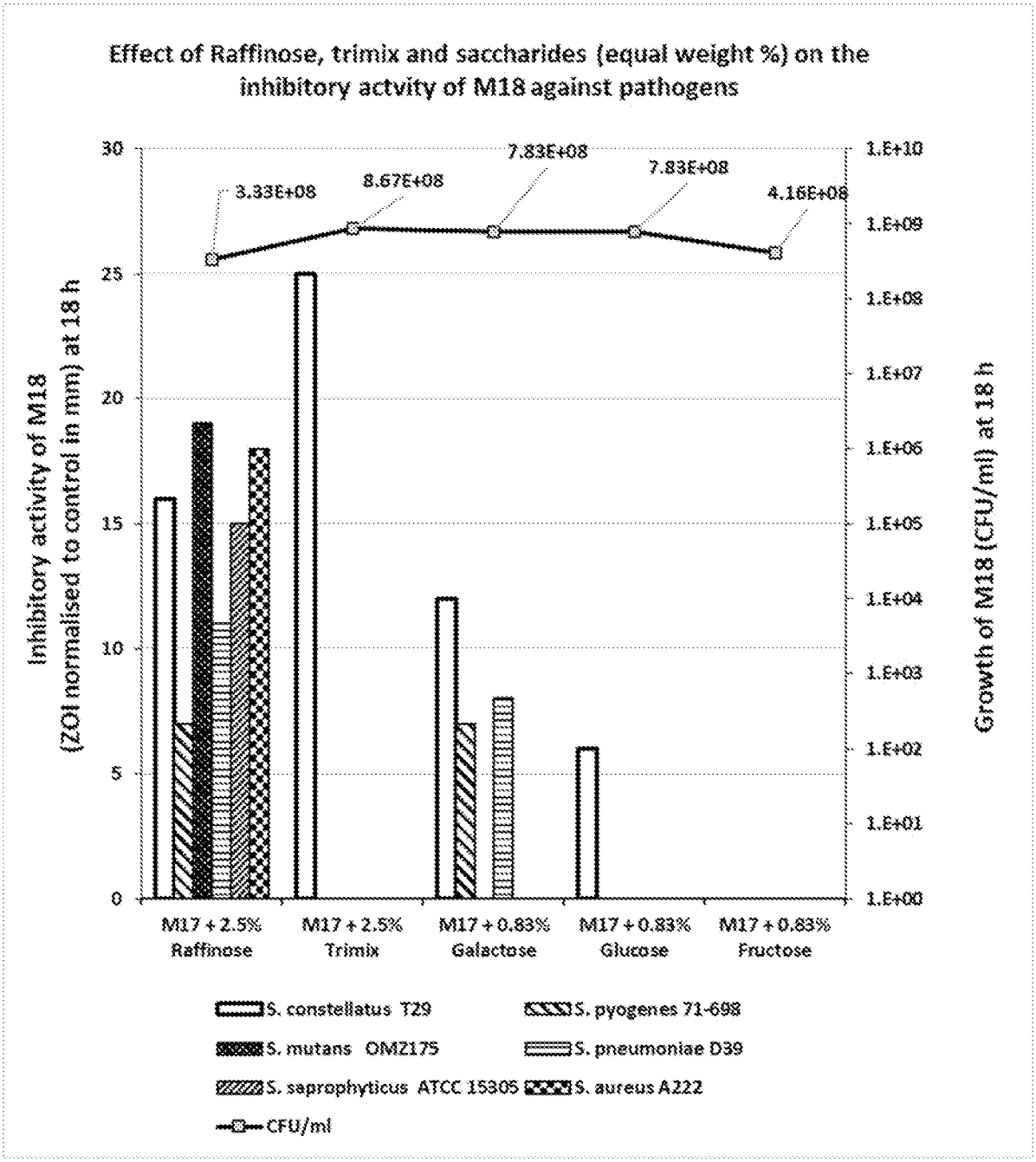

Effect of Raffinose, trimix and saccharides (equal weight %) on the inhibitory actvity of M18 against pathogens
Inhibitory activity of M18 (ZOI normalised to control in mm) at 18 h
Growth of M18 (CFU/ml) at 18 h
3.33E+08
8.67E+08
7.83E+08
7.83E+08
4.16E+08
M17 + 2.5% Raffinose
M17 + 2.5% Trimix
M17 + 0.83% Galactose
M17 + 0.83% Glucose
M17 + 0.83% Fructose
S. constellatus T29
S. pyogenes 71-698
S. mutans OMZ175
S. pneumoniae D39
S. saprophyticus ATCC 15305
S. aureus A222
CFU/ml

Figure 29

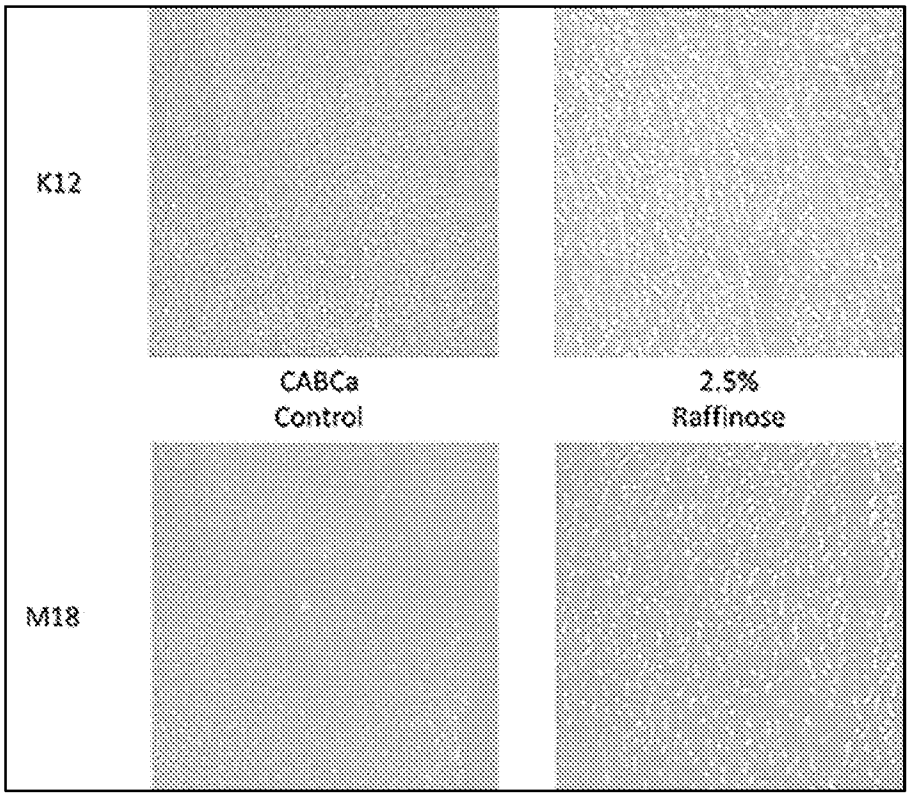


Figure 30

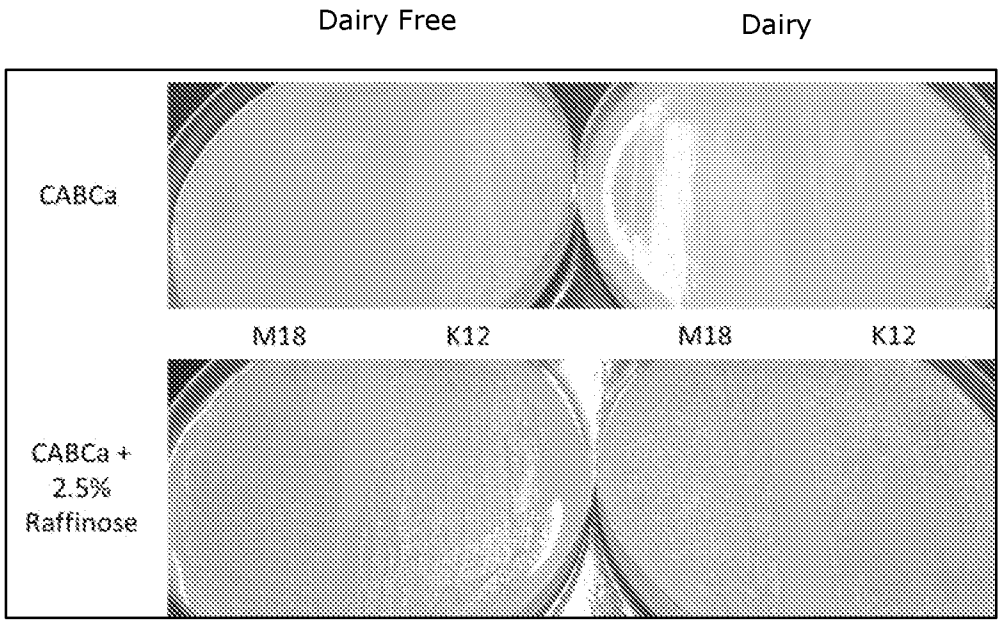


Figure 31

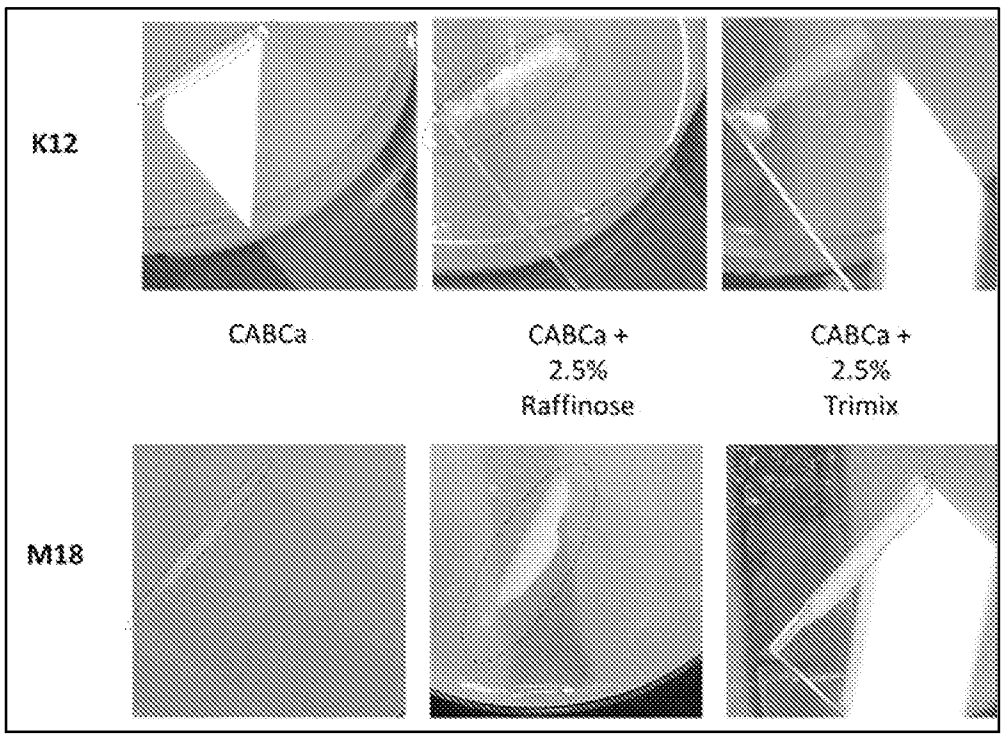


Figure 32

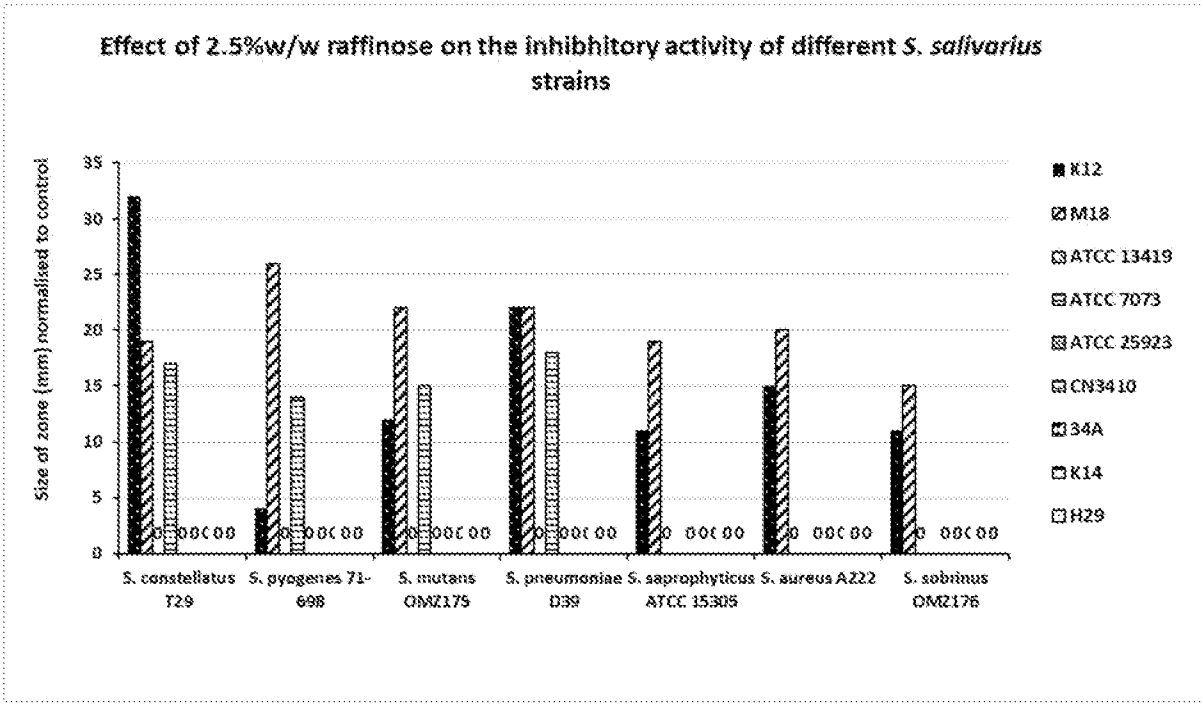


Figure 33

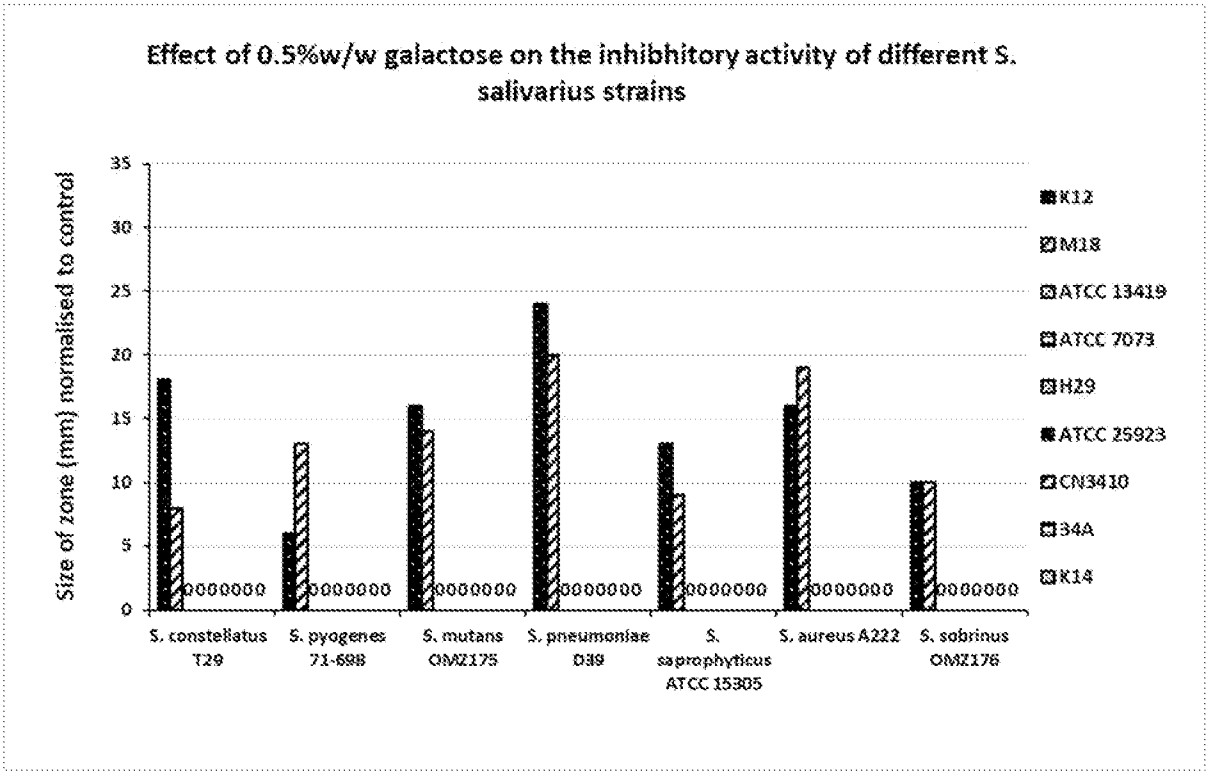


Figure 34

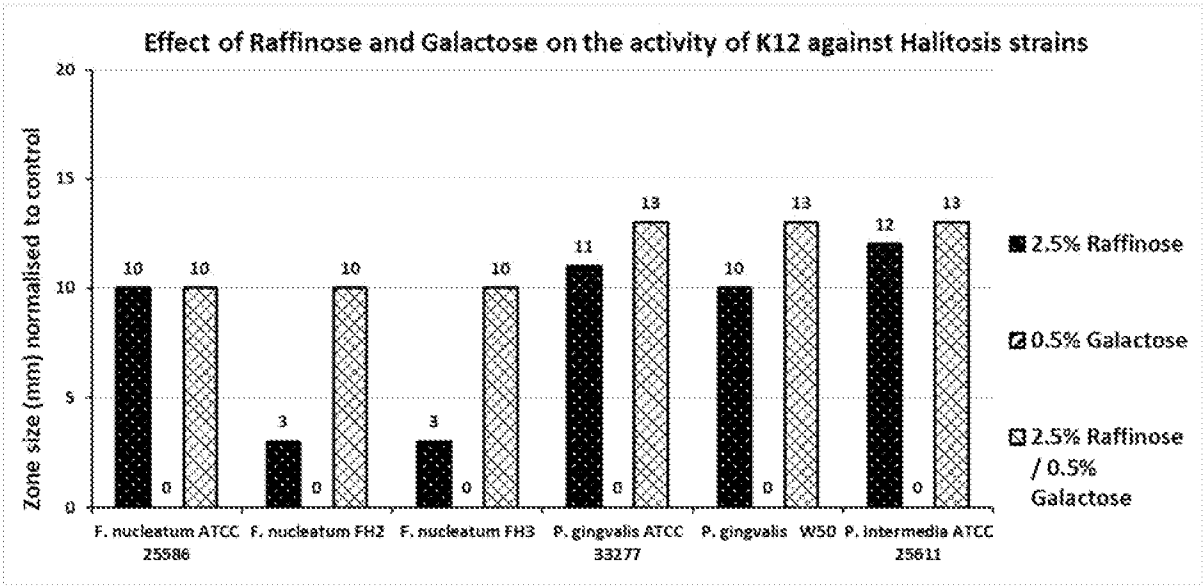


Figure 35

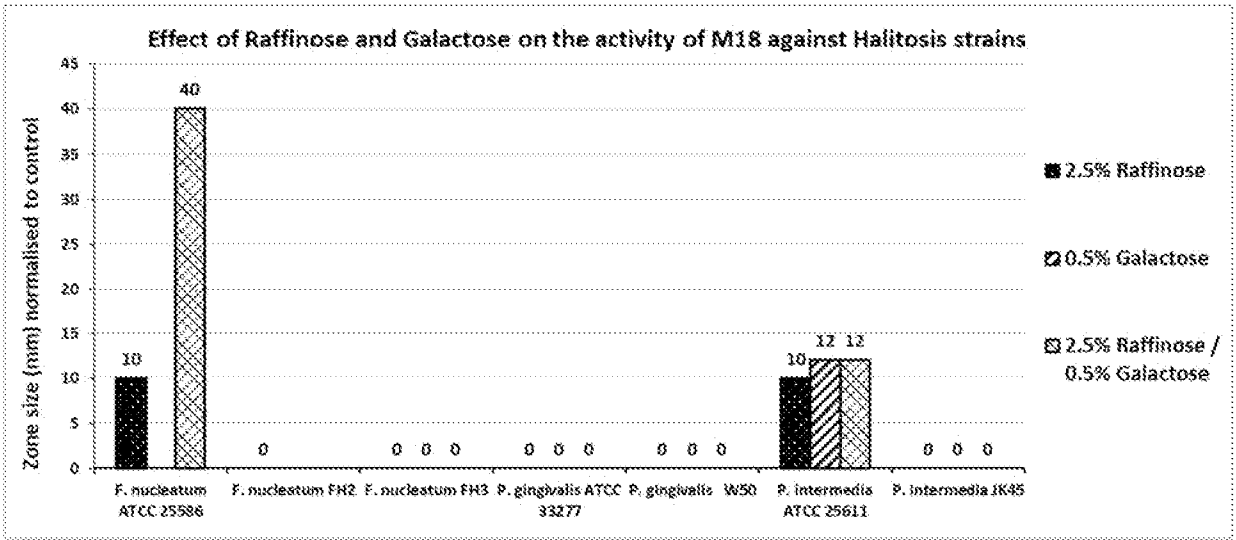


Figure 36

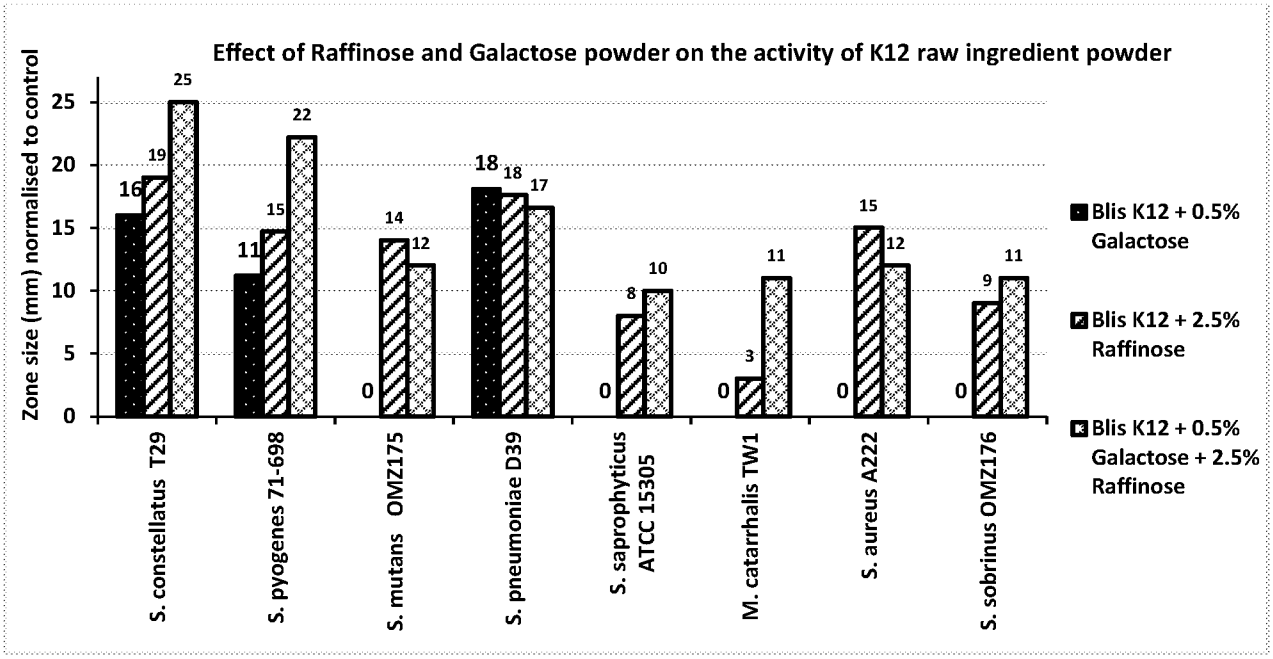


Figure 37

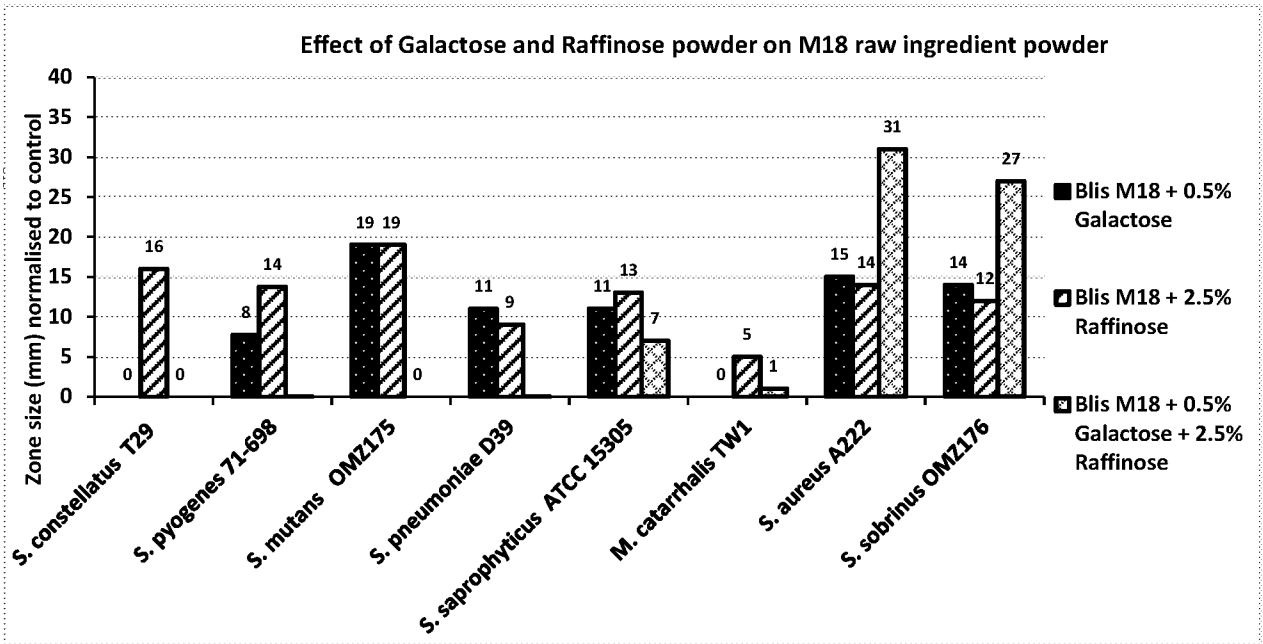
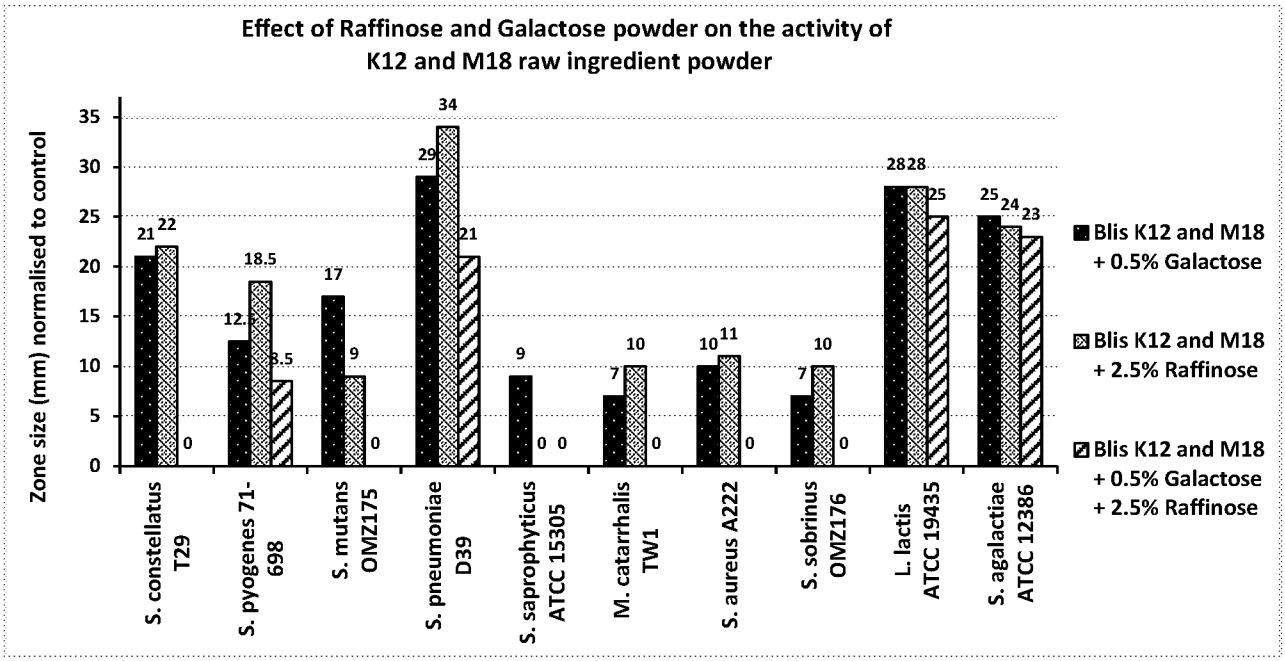


Figure 38


Effect of Raffinose and Galactose powder on the activity of K12 and M18 raw ingredient powder
Zone size (mm) normalised to control
0
5
10
15
20
25
30
35
21
22
0
12.
18.5
8.5
17
9
0
29
34
21
9
0
0
7
10
0
10
11
0
7
10
0
28
28
25
25
24
23
S. constellatus T29
S. pyogenes 71-698
S. mutans OMZ175
S. pneumoniae D39
S. saprophyticus ATCC 15305
M. catarrhalis TW1
S. aureus A222
S. sobrinus OMZ176
L. lactis ATCC 19435
S. agalactiae ATCC 12386
Blis K12 and M18 + 0.5% Galactose
Blis K12 and M18 + 2.5% Raffinose
Blis K12 and M18 + 0.5% Galactose + 2.5% Raffinose

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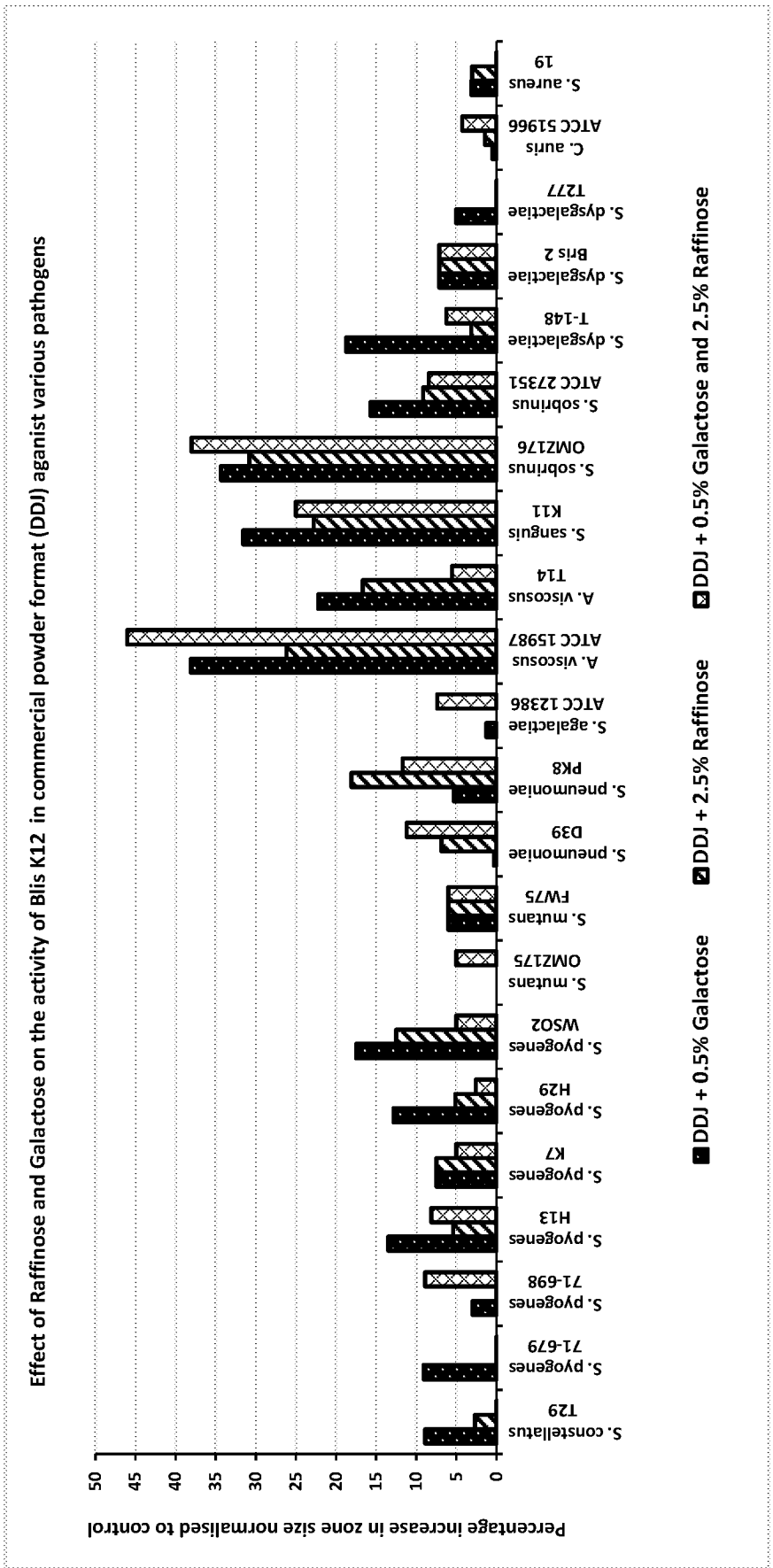

Figure 39.

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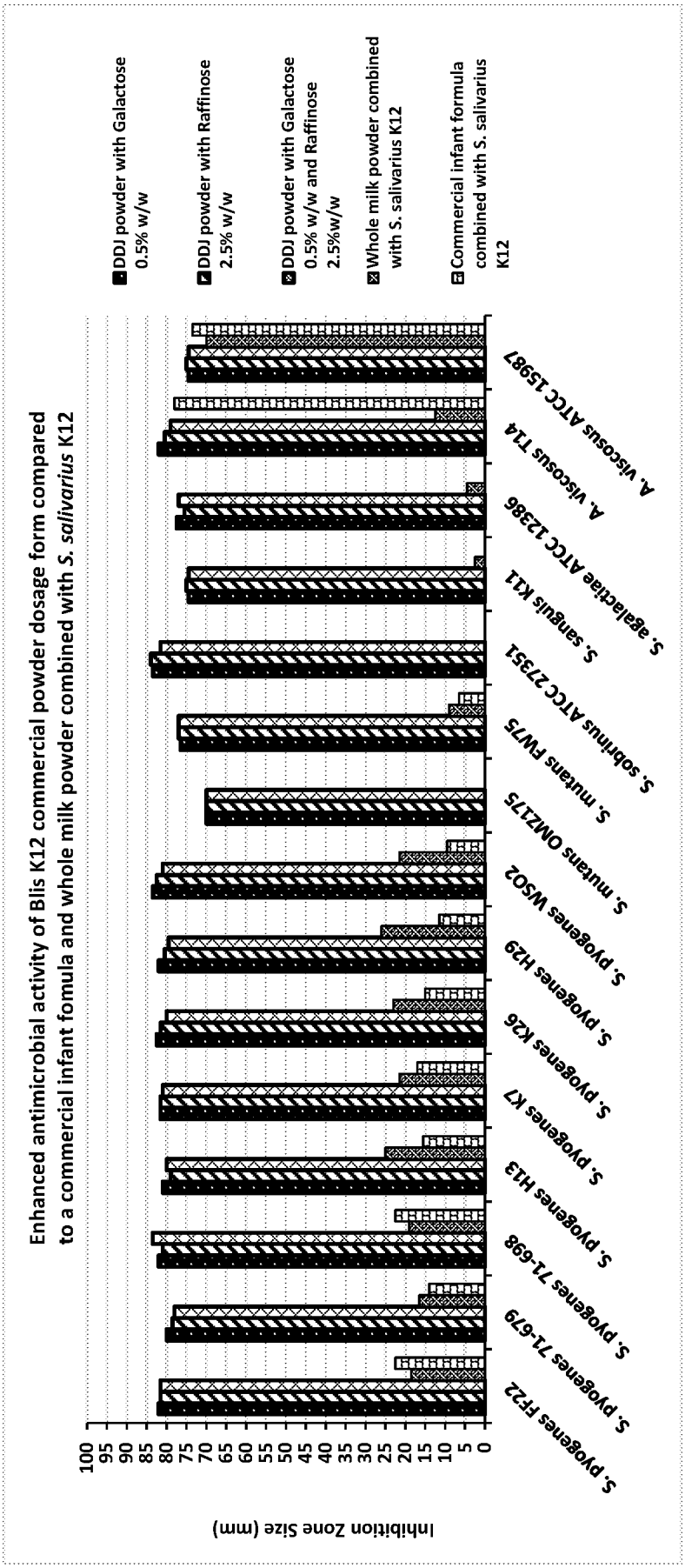


Figure 40.

Figure 41.

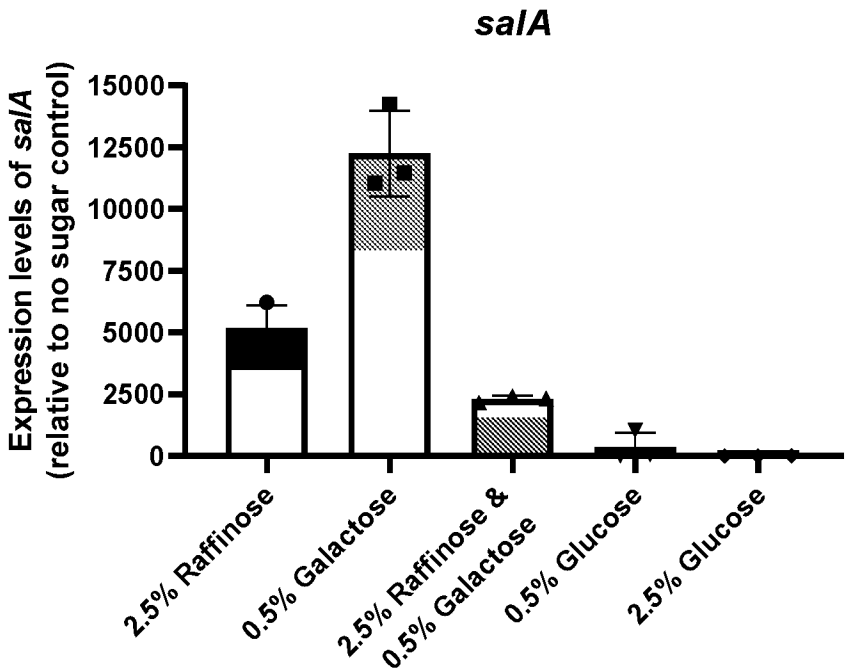


Figure 42.

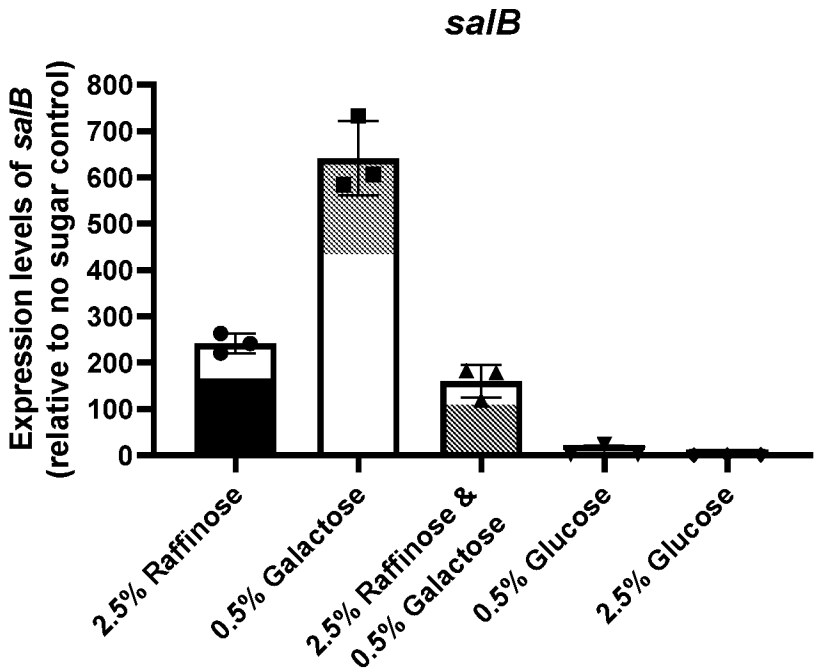


Figure 43.

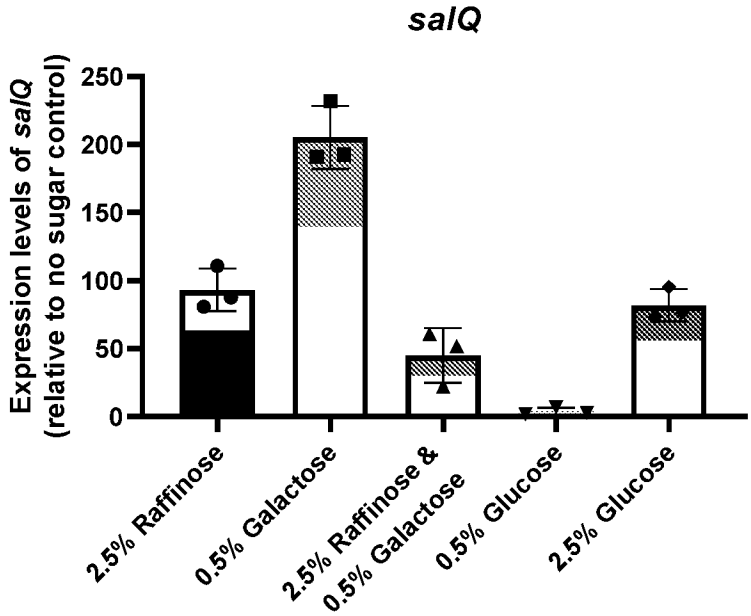


Figure 44.

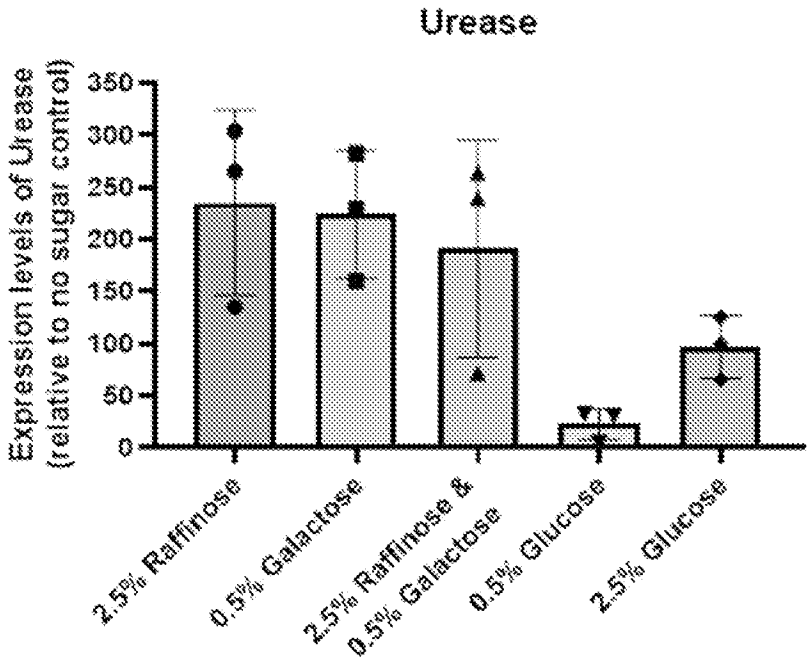
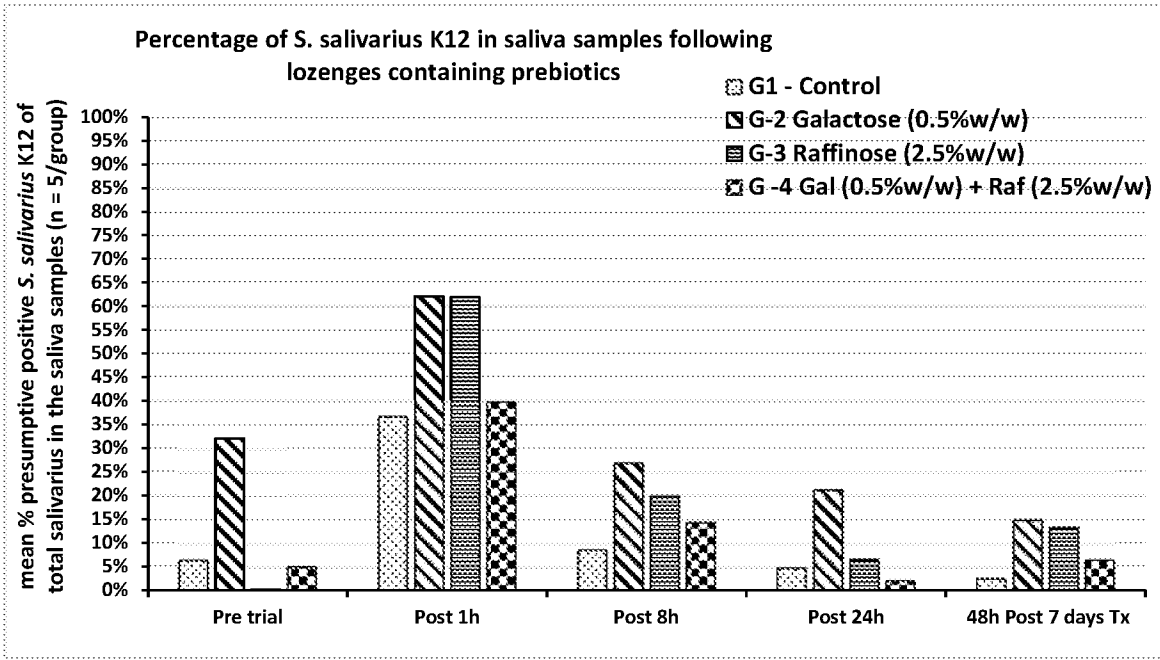


Figure 45.


Percentage of S. salivarius K12 in saliva samples following lozenges containing prebiotics
G1 - Control
G-2 Galactose (0.5%w/w)
G-3 Raffinose (2.5%w/w)
G -4 Gal (0.5%w/w) + Raf (2.5%w/w)
mean % presumptive positive S. salivarius K12 of total salivarius in the saliva samples (n = 5/group)
100%
95%
90%
85%
80%
75%
70%
65%
60%
55%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Pre trial
Post 1h
Post 8h
Post 24h
48h Post 7 days Tx

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2022/059321

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/744 (2015.01) A61P 31/04 (2006.01) A61K 47/26 (2006.01) C12R 1/46 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

PATENW (English full text, WPIAP, EPODOC), MEDLINE, CAPLUS, BIOSIS, EMBASE FSTA, NAPRALERT: Keywords – BLIS, galactose, raffinose, probiotic, prebiotic and like terms. Applicant/Inventor search: Patentscope, Google Patents/Scholar, PubMed, IP Australia internal databases - BLIS TECHNOLOGIES LIMITED, JOHN ROBERT TAGG, JOHN DAVID FRANCIS HALE, ROHIT JAIN, NICOLA CHRISTINE JONES.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] 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

"D" document cited by the applicant in the international application

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 2022	22 December 2022

Name and mailing address of the ISA/AU

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Authorised officer

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/IB2022/059321**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/0343899 A1 (KNOZE JR. CORPORATION) 14 November 2019 [0024]-[0026], [0044]	1-85
X	US 2018/0280430 A1 (BALCHEM CORPORATION) 04 October 2018 [0018]-[0020], [0084]	1-85
X	WO 2017/129639 A1 (NESTEC S.A.) 03 August 2017 p10-p11, p16, l28-p17, l2, p20	1, 21-32, 44-63 and 71-85
X	US 2019/0070229 A1 (HAUDONGCHUN CO., LTD.) 07 March 2019 Abstract and Discussion	1-85
X	BINGYONG MAO, et al "*In vitro* fermentation of raffinose by the human gut bacteria." Food and Function, Vol 9, p5824–5831. 2018. Abstract and Discussion	1-20, 24-29,44-56, 73-84
X	ZARTL, B. et al "Fermentation of non-digestible raffinose family oligosaccharides and galactomannans by probiotics" Food and Function, Vol. 9, p1638-1646. 2018 Abstract, Discussion, table 2	1-20, 24-29,44-56, 73-84
A	Cherry Flavoured Bio Yogurt by Valio. Mintel GNPD database. Dec 2013. https://www.gnpd.com/sinatra/recordpage/2253814/from_search/0z1QqqLlNw/?page=1	
A	Innerbio-Formula bu NOEVIR. Mintel GNPD database. July 2004. https://www.gnpd.com/sinatra/recordpage/281180/from_search/tre7pOr7TB/?page=7	
A	WEAVER, C. A. et al "Inactivation of the ptsl gene encoding enzyme 1 of the sugar phphotransferase system of *Streptococcus salivarius:* effects of growth and urease expression." Microbiology, Vol. 146, page 1179-1185. 2000.	

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

   Sequence Listing was not used for searching purposes.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/IB2022/059321**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2019/0343899 A1	14 November 2019	US 2019343899 A1	14 Nov 2019
		US 11083759 B2	10 Aug 2021
		AU 2018257624 A1	05 Dec 2019
		AU 2018257624 B2	08 Jul 2021
		CN 110520119 A	29 Nov 2019
		EP 3609488 A1	19 Feb 2020
		JP 2020517746 A	18 Jun 2020
		JP 2021167338 A	21 Oct 2021
		KR 20190141707 A	24 Dec 2019
		KR 20220034925 A	18 Mar 2022
		US 9795579 B1	24 Oct 2017
		US 2018303782 A1	25 Oct 2018
		US 10398670 B2	03 Sep 2019
		US 2019083435 A1	21 Mar 2019
		US 10471033 B2	12 Nov 2019
		US 2019343900 A1	14 Nov 2019
		US 11083760 B2	10 Aug 2021
		US 2018303781 A1	25 Oct 2018
		US 11364217 B2	21 Jun 2022
		US 2021338747 A1	04 Nov 2021
		US 2022304967 A1	29 Sep 2022
		WO 2018200223 A1	01 Nov 2018
		WO 2019055665 A1	21 Mar 2019
US 2018/0280430 A1	04 October 2018	US 2018280430 A1	04 Oct 2018
		AU 2016330886 A1	29 Mar 2018
		BR 112018006368 A2	09 Oct 2018
		CA 2997680 A1	06 Apr 2017
		EP 3355989 A1	08 Aug 2018
		MX 2018003926 A	06 Sep 2018
		WO 2017059101 A1	06 Apr 2017
WO 2017/129639 A1	03 August 2017	WO 2017129639 A1	03 Aug 2017
		AU 2017211953 A1	10 May 2018
		CN 108495634 A	04 Sep 2018
		EP 3407897 A1	05 Dec 2018
		MX 2018008955 A	17 Jan 2019

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		PH 12018501325 A1	18 Feb 2019
		RU 2018130488 A	27 Feb 2020
		US 2021205341 A1	08 Jul 2021
US 2019/0070229 A1	07 March 2019	US 2019070229 A1	07 Mar 2019
		AU 2017264267 A1	12 Jul 2018
		BR 112018070277 A2	29 Jan 2019
		CA 3010577 A1	16 Nov 2017
		CN 108883127 A	23 Nov 2018
		EP 3454869 A1	20 Mar 2019
		JP 2019524636 A	05 Sep 2019
		KR 101784847 B1	13 Oct 2017
		MX 2018010994 A	07 Mar 2019
		PH 12018501603 A1	15 May 2019
		RU 2018124654 A	10 Jun 2020
		WO 2017196006 A1	16 Nov 2017

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.