BLIS Technologies

July 26, 2018

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re GRAS Exemption Claim for *Streptococcus salivarius* M18

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, BLIS Technologies Ltd. hereby informs the U.S. Food and Drug Administration (FDA) of the conclusion that Streptococcus salivarius M18, is Generally Recognized as Safe (GRAS) for its intended conditions of use in food as described in the enclosed notice, and therefore is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

I verify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection Virus and Spyware Protection.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Brian Watson
CEO
BUS Technologies Ltd.
81 Glasgow Street
South Dunedin,
Dunedin 9012
New Zealand
Brian.watson@blis.co.nz

GRAS Notice for *Streptococcus Salivarius* M18

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, BUS Technologies Ltd. (BUS) hereby informs the U.S. Food and Drug Administration (FDA) that *Streptococcus salivarius* M18 (5. *salivarius* M18), as manufactured by BUS, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on BUS's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of BLIS, Brian Watson hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to BUS and pertinent to the evaluation of the safety and GRAS status of 5. *sa/ivarius* M18 as an ingredient for addition to food.

Signed,

[redacted]

27/07/18

Brian Watson
CEO
Brian.watson@blis.co.nz

Date

1.1 Name and Address of Notifier

BLIS Technologies Ltd.
81 Glasgow Street
South Dunedin
Dunedin 9012
New Zealand

1.2 Common Name of Notified Substance

Streptococcus salivarius M18

1.3 Conditions of Use

BUS Technologies Ltd. (BUS) intends to market a freeze-dried powder of 5. *salivarius* M18 as a food ingredient in the United States (U.S.) for use in a variety of conventional food and beverage products *[i.e.,* baby, infant, and toddler foods (excluding infant formula); baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices; soft candy; sweet sauces, toppings, and syrups] at a level of 20 mg per seNing (providing a minimum of $1x10^9$ colony-forming units [CFU]/serving). The individual proposed food-uses and use-levels for 5. *salivarius* M18 employed in the current intake analysis are summarized in Table 1.3-1. Food codes representative of each proposed food-use were chosen

GRAS NOTICE FOR *STREPTOCOCCUS SALIVARIUS* M18

PREPARED FOR:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
BLIS Technologies Ltd.
81 Glasgow Street
South Dunedin
Dunedin 9012
New Zealand

DATE:
26 July 2018

GRAS Notice for *Streptococcus Salivarius* M18

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GRAS Notice for *Streptococcus Salivarius* M18

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, BLIS Technologies Ltd. (BLIS) hereby informs the U.S. Food and Drug Administration (FDA) that *Streptococcus salivarius* M18 (*S. salivarius* M18), as manufactured by BLIS, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on BLIS's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of BLIS, Brian Watson hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to BLIS and pertinent to the evaluation of the safety and GRAS status of *S. salivarius* M18 as an ingredient for addition to food.

Signed,

Brian Watson
CEO
Brian.watson@blis.co.nz

Date

1.1 Name and Address of Notifier

BLIS Technologies Ltd.
81 Glasgow Street
South Dunedin,
Dunedin 9012
New Zealand

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Streptococcus salivarius M18

1.3 Conditions of Use

BLIS Technologies Ltd. (BLIS) intends to market a freeze-dried powder of *S. salivarius* M18 as a food ingredient in the United States (U.S.) for use in a variety of conventional food and beverage products [*i.e.,* baby, infant, and toddler foods (excluding infant formula); baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices; soft candy; sweet sauces, toppings, and syrups] at a level of 20 mg per serving (providing a minimum of $1x10^9$ colony-forming units [CFU]/serving). The individual proposed food-uses and use-levels for *S. salivarius* M18 employed in the current intake analysis are summarized in Table 1.3-1. Food codes representative of each proposed food-use were chosen

from the National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Surveys (NHANES) for the years 2003-2004 (NHANES 2003-2004) and 2005-2006 (NHANES 2005-2006) (CDC, 2006; CDC, 2009; USDA, 2009). Food codes were grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (CFR) (U.S. FDA, 2016).

Table 1.3-1 Food-Uses and Use-Levels for *Streptococcus salivarius* M18

Food Category	Proposed Food-Uses	*S. salivarius* M18 Use-Level		Serving Size	Use-Level
Baby and Toddler Foods	Cereals, Baby Food	$1.0x10^9$	20	15 (dry, instant)[a] 110 (RTS)[a]	0.13 (dry, instant) 0.018 (RTS)
	Cookies, Crackers, and Puffs, Baby/Toddler Food	$1.0x10^9$	20	7[a]	0.10
	RTS Fruit-Based Baby/Toddler Food	$1.0x10^9$	20	60 (strained)[a] 110 (junior)[a] 125 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.016 (toddler)
	Fruit Juices, Baby Food	$1.0x10^9$	20	125[a]	0.016
	RTS Dinners, Baby/Toddler Food	$1.0x10^9$	20	60 (strained)[a] 110 (junior)[a] 170 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.012 (toddler)
	RTS Desserts, Baby Food	$1.0x10^9$	20	60 (strained) 110 (junior)	0.03 (strained) 0.018 (junior)
	RTF Vegetable-Based Baby/Toddler Food	$1.0x10^9$	20	60 (strained) 110 (junior) 70 (toddler)	0.03 (strained) 0.018 (junior) 0.029 (toddler)
Baked Goods and Baking Mixes	Cookies (chocolate coating)	$1.0x10^9$	20	20	0.10
Beverages and Beverage Bases	Meal Replacement powders (fortified, protein, and mineral replenish)	$1.0x10^9$	20	16 to 40	0.05 to 0.13
	Sports and Energy Drinks	$1.0x10^9$	20	250	0.01
	Water (still or mineral)	$1.0x10^9$	20	237	0.01
Breakfast Cereals	Breakfast Cereals	$1.0x10^9$	20	29	0.07
	Muesli and Dry Blended Cereals	$1.0x10^9$	20	85	0.02
Cheeses	Natural Cheeses	$1.0x10^9$	20	20 to 30	0.07 to 0.10
Chewing Gum	Chewing Gum	$1.0x10^9$	20	3	0.67
Dairy Product Analogues	Milk Substitutes	$1.0x10^9$	20	244	0.01
Frozen Dairy Desserts and Mixes	Frozen Yogurt	$1.0x10^9$	20	174	0.02
	Ice Cream	$1.0x10^9$	20	66	0.03
Gelatins, Puddings, and Fillings	Custards (pourable)	$1.0x10^9$	20	113	0.02
	Dessert Mixes (powder)	$1.0x10^9$	20	25	0.08
Grain Products and Pastas	Granola and Breakfast Bars	$1.0x10^9$	20	28	0.07
	Protein Bars	$1.0x10^9$	20	68	0.03
Hard Candy	Mint Candies	$1.0x10^9$	20	25	0.08
Milk, Whole and Skim	Milk (flavored, pasteurized)	$1.0x10^9$	20	244	0.01
	Milk (fresh)	$1.0x10^9$	20	244	0.01
	Milk Powder (skim or whole)	$1.0x10^9$	20	23 to 32	0.06 to 0.09

Table 1.3-1 Food-Uses and Use-Levels for *Streptococcus salivarius* M18

Food Category	Proposed Food-Uses	*S. salivarius* M18 Use-Level		Serving Size (g or mL)*	Use-Level (%)
		CFU/serving	mg/serving		
Milk Products	Cream (pasteurized)	$1.0x10^9$	20	244	0.01
	Cultured Milk Products	$1.0x10^9$	20	180	0.01
	Dairy Desserts	$1.0x10^9$	20	100 to 180	0.01 to 0.02
	Milkshake Mixes (powder)	$1.0x10^9$	20	21	0.10
	Yogurt	$1.0x10^9$	20	227	0.01
	Yogurt Drinks	$1.0x10^9$	20	244	0.01
Nuts and Nut Products	Peanut Butter	$1.0x10^9$	20	32	0.06
Processed Fruits and Fruit Juices	Fruit-Flavored Beverages (powder)	$1.0x10^9$	20	18	0.11
	Fruit Juices	$1.0x10^9$	20	263	0.01
	Fruit Juice Drinks	$1.0x10^9$	20	209	0.01
Soft Candy	Chewable Lozenges	$1.0x10^9$	20	3	0.67
	Chocolate Bars	$1.0x10^9$	20	44	0.05
	Soft Gel and Rapid Melt Technologies	$1.0x10^9$	20	2	1
Sweet Sauces, Toppings, and Syrups	Cinnamon, Nutmeg, and Chocolate Sprinkle	$1.0x10^9$	20	4[a]	0.50
	Sugar and Sweetener Sprinkle	$0.5x10^9$	10	4[a]	0.25

CFU = colony-forming unites; RTF = ready to feed; RTS = ready to serve.

* Serving sizes were provided by BLIS Technologies Ltd., unless otherwise indicated.

[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the United States Code of Federal Regulations (21 CFR §101.12).

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a) and (b) of the CFR (U.S. FDA, 2016), *S. salivarius* M18 manufactured by BLIS has been concluded to have GRAS status for use as an ingredient for addition to specified conventional food and beverage products, as described in Table 1.3-1, on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be made available to the U.S. FDA for review and copying upon request during business hours at the offices of:

BLIS Technologies Ltd.
81 Glasgow Street
South Dunedin
Dunedin 9012
New Zealand

In addition, should the U.S. FDA have any questions or additional information requests regarding this Notification during or after the Agency's review of the notice, BLIS will supply these data and information.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is BLIS's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. 552.

Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

The ingredient that is the subject of this GRAS determination is a *S. salivarius* M18 preparation, an ingredient containing a proprietary strain maintained by BLIS and grown under controlled conditions in sterile fermentation vessels. Additional description of the ingredient and information characterizing the identity of the organism is presented in the following sections below.

BLIS Technologies notes that the related strain *S. salivarius* K12 has been previously concluded to have GRAS status for select food and beverage uses (GRAS Notice [GRN] 591). This strain has been subject to comprehensive safety evaluations by a number of authoritative food safety bodies including the U.S. FDA, Health Canada, and Food Standards Australia New Zealand and has been concluded to be safe for food use. Based on the GRAS status and history of use of strain K12 in food, *S. salivarius* K12 is used as a reference strain for the safety evaluation of *S. salivarius* M18. Where applicable comparisons of M18 to K12 are presented throughout the notice as they apply to the safety assessment of M18.

2.1.1 Trade Name

BLIS M18

2.1.2 Taxonomic Lineage

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Lactobacillales
Family: Streptococcaceae
Genus: *Streptococcus*
Species: *salivarius*
Strain: M18

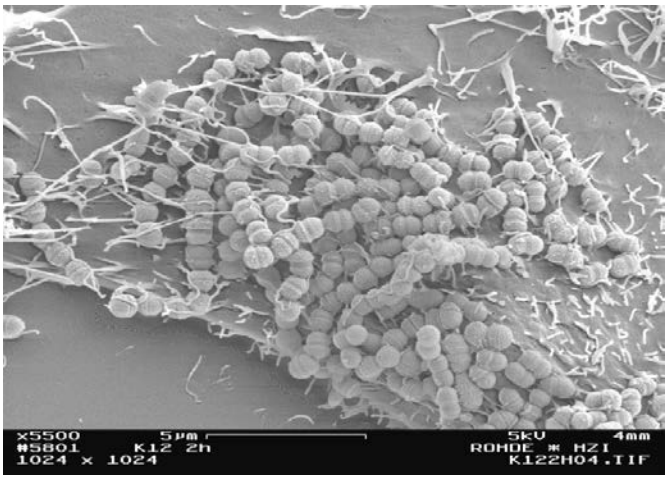
2.1.3 History

S. salivarius strain BLIS M18 was isolated from the oral cavity of a healthy adult human. *S. salivarius* M18 has been deposited in the American Type Culture Collection (ATCC) as ATCC BAA 2593. It also has been logged with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkuturen [DSMZ] GmbH) under the accession number DSMZ 14685. The species and strain identity have been characterized using the most current phenotypic and genotypic techniques, as discussed in Sections 2.1.4 and 2.1.5 below.

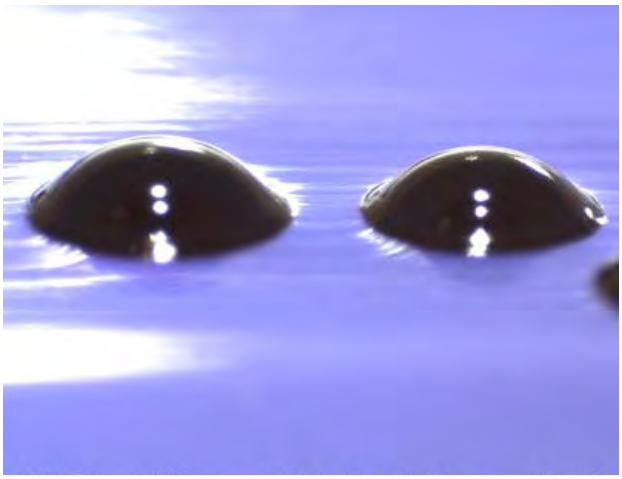
2.1.4 Phenotypic Identity

Streptococci are spherical, non-motile, Gram-positive microorganisms that occur in chains or pairs (Figure 2.1.4-1). Typically, the identification of *S. salivarius* M18 from a sample (heterogeneous bacteriological sample) starts with selection on semi-selective bacteriological agar (Mitis-Salivarius Agar). *S. salivarius* distinctive colony types on the agar are then isolated and repropagated on non-specific bacteriological media for further identification procedures. These procedures include conventional biochemical techniques such as the API 20 Strep and API 50 CH tests described below. On agar plates, the 2 strains *S. salivarius* M18 and *S. salivarius* K12 appeared identical, but further characterization revealed subtle differences in their carbohydrate fermentation profiles, enzymatic makeup, and antimicrobial production, as described in the sections below.

Figure 2.1.4-1 Colony Morphology of *Streptococcus salivarius* sp.



A) Electron micrograph of *S. salivarius* K12, representative of *S. salivarius* M18.



B) Growth of *S. salivarius* sp. on Mitis-Salivarius agar results in raised blue colonies.

2.1.4.1 Carbohydrate Fermentation

The sugar fermentation profile of *S. salivarius* M18 was determined using the API 50 CH test system (bioMérieux) and is presented in Table 2.1.4.1-1. The test system, which analyzes 49 different sugars to determine carbohydrate metabolism. When compared with *S. salivarius* K12, differences in the fermentation of only a few substrates were noted, including L-arabinose, D-melibiose, glycogen, gentiobiose, amygdalin, and D-tagatose.

Table 2.1.4.1-1 API 50 CH Carbohydrate Fermentation Profile of *Streptococcus salivarius* M18 and K12 (24 hours at 37°C)

Substrate	M18	K12	Substrate	M18	K12
Glycerol	-	-	Salicin	+	+
Erythritol	-	-	D-cellobiose	+	+
D-arabinose	-	-	D-maltose	+	+
L-arabinose	+ (anaerobic only)	-	D-lactose	+	+
D-ribose	-	-	D-melibiose	+ (aerobic only)	-
D-xylose	-	-	D-saccharose	+	+
L-xylose	-	-	D-trehalose	+	+
D-adonitol	-	-	Inulin	+	+
Methyl-βD-xylopranoside	-	-	D-melezitose	-	-
D-galactose	+	+	D-raffinose	+	+
D-glucose	+	+	Amidon	-	-
D-fructose	+	+	Glycogen	+ (anaerobic only)	-
D-mannose	+	+	Xylitol	-	-
L-sorbose	-	-	Gentiobiose	+	-
L-rhamnose	-	-	D-turanose	-	-
Dulctiol	-	-	D-lyxose	-	-
Inositol	-	-	D-tagatose	+ (anaerobic only)	+
D-mannitol	-	-	D-fucose	-	-
D-sorbitol	-	-	L-fucose	-	-
Methyl-αD-mannopyranoside	-	-	D-arabitol	-	-
Methyl-αD-glucopyranoside	-	-	L-arabitol	-	-
N-acetylglucosamine	+	+	Gluconate	-	-
Amygdalin	+	-	2-ketogluconate	-	-
Arbutin	+	+	5-ketogluconate	-	-
Esculin	+	+			

2.1.4.2 Enzyme Profile

The enzyme profile of *S. salivarius* M18 was determined using the API 20 Strep test system (bioMérieux), which includes 20 wells containing the following biochemical tests in dehydrated form: 3 classical biochemical tests (acetoin production, hippurate hydrolysis, and arginine hydrolase); 4 oxidase reactions (β-glucosidase, β-glucuronidase, β-galactosidase, and α-galactosidase); 1 arylamidase reaction (pyrrolidonyl); and 9 carbon substrate fermentation (ribose, arabinose, mannitol, sorbitol, lactose, inulin, raffinose, starch, and glycogen). The detection for hemolysis is an extra test determined by streaking the cultures on human blood agar plates. As presented in Table 2.1.4.2-1, *S. salivarius* M18 tested positive for the following: acetoin production, β-glucosidase, α-galactosidase, β-galactosidase, leucine aminopeptidase, D-lactose, D-trehalose, inulin, and D-raffinose. Enzymatic features identified in *S. salivarius* M18 and absent in the K12 strain included α- and β-galactosidase enzyme activity. Neither strain displayed β-hemolytic activity. BLIS notes that α- and β-galactosidase enzyme activity is a common phenotype of many bacteria strains indigenous to the gastrointestinal tract.

Table 2.1.4.2-1 API 20 Strep Enzyme Profile for *Streptococcus salivarius* M18 and K12 (24 hours at 37°C)

Enzyme Reaction	*S. salivarius* M18	*S. salivarius* K12
Acetoin production	+	+
Hippuric acid hydrolysis	-	-
β-Glucosidase	+	+
Pyrrolidonyl arylamidase	-	-
α-Galactosidase	+	-
β-Glucuronidase	-	-
β-Galactosidase	+	-
Alkaline phosphatase	-	+
Leucine aminopeptidase	+	+
Arginine dihydrolase	-	-
D-ribose	-	-
L-arabinose	-	-
D-mannitol	-	-
D-sorbitol	-	-
D-lactose	+	+
D-trehalose	+	+
Inulin	+	+
D-raffinose	+	+
Starch	-	-
Glycogen	-	-
β-Hemolysis	-	-

2.1.4.2.1 Dextranase

Certain bacteria, including select strains of *S. salivarius*, produce enzymes known as dextranases that degrade dextran and release sugar subunits into the oral cavity. Dextranases have been shown to reduce the levels of mutans streptococci biofilms by up to 18% (Delisle, 1976; Hayacibara *et al.,* 2004) and also reduce the number of potential binding sites for mutans streptococci bacteria. A survey conducted by BLIS looking for dextranase-producing *S. salivarius* found that only a few strains, including *S. salivarius* M18 and *S. salivarius* K12, were capable of producing dextranase.

2.1.4.2.2 Urease

Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide and is believed to provide a buffering capacity that protects the strain from low pH environments. *S. salivarius* strains were tested by BLIS for their ability to produce urease when grown on Christensens agar. *S. salivarius* M18 was identified as having an ureolytic phenotype. Similarly, *S. salivarius* K12 has also been identified as being ureolytic (Guglielmetti *et al.,* 2010). Although urease activity has historically been viewed as a virulence phenotype in microorganisms, this generic designation is no longer considered appropriate as the phenotype has been detected in Bifidobacteria and lactic acid 'probiotic' bacteria and appears to be a characteristic trait of *S. salivarius* and other species that are positively associated with human health (Mora and Arioli, 2014).

2.1.4.3 Production of Antimicrobials (Bacteriocin-Like Inhibitory Substances)

The production of bacteriocins by naturally occurring oral streptococci is widespread. *S. salivarius* M18 produces 4 bacteriocins: the type II lantibiotics: salivaricins A2, 9, MPS, and M (Heng *et al.,* 2011; Wescombe *et al.,* 2011). In comparison, *S. salivarius* K12 only produces 2 bacteriocins: salivaricin A2 and salivaricin B (Wescombe *et al.*, 2006).

The ability to identify bacteriocin production can be determined using the deferred antagonism test (Tagg and Bannister, 1979). In this test, the producer strain secretes the bacteriocin(s) onto the bacterial growth medium (agar) and following this, various bacterial indicator strains are applied to this medium. If the bacteriocin inhibits the indicator strain, then regions of no growth are seen on the agar. Using this method, bacteria can be identified and classified by their 'bacteriocin fingerprints'—also known as P-typing (Tagg and Bannister, 1979). Deferred antagonism tests with *S. salivarius* M18 demonstrated that this strain can inhibit a wide range of oral bacteria, in particular *S. mutans.* A comparison of anti-*S. mutans* inhibitory activity by *S. salivarius* K12 found that *S. salivarius* M18 was able to inhibit more strains (Table 2.1.4.3-1) and this is believed to be due to the production of the extra bacteriocin, salivaricin M. Further discussion of the bacteriocin profile of M18 as it pertains to the safety of the organism for use as a food ingredient are presented in Section 6.7.1.

Table 2.1.4.3-1 Comparison of *Streptococcus salivarius* M18 and K12 Inhibitory Profiles against *S. mutans*

Producer Strain	Test Medium	Inhibition of *S. mutans* Strain								
		ATCC 10449	OMZ175	H7	13M	K56	K60	M46	MutI	MutII
M18	TSBCa	++	+++	+	-	-	-	+	+	-
M18	TSBCaYE	+++	+++	++	+	+	+	++	+	+
K12	TSBCa	++	++	-	-	-	-	++	-	-
K12	TSBCaYE	++	++	++	-	+	+	++	+	-

ATCC = American Type Culture Collection.

2.1.5 Genotypic Characterization

2.1.5.1 Genome Sequencing and Species Characterization

The *S. salivarius* M18 genome, consisting of bacterial chromosome and its 180 kb megaplasmid pSsal-M18, was sequenced by whole-genome shotgun strategy (Heng *et al.,* 2011). The high-quality draft *S. salivarius* M18 chromosome sequence currently comprises 5 supercontigs (2,142,944 base pairs; guanine-cytosine [GC] content of 39.6%). Putative chromosomal contigs were ordered relative to the megaplasmid-free *S. salivarius* CCHSS3 genome sequence (GenBank accession number FR873481), and gap closures conducted by direct Sanger-based sequencing of polymerase chain reaction (PCR) amplicons generated with specific primers designed for contig termini. Sequencing of pSsal-M18 was the first example of a fully sequenced streptococcal megaplasmid and it was resolved to be 183,037 base pairs long (GC content 34.87%). Of the 172 identified protein-coding sequences in the *S. salivarius* M18 megaplasmid, 16 were found to belong to known bacteriocin encoding loci for salivaricins A, 9, and MPS. A locus (*slm*) was also identified on the *S. salivarius* M18 chromosome encoding a new lantibiotic bacteriocin with anti-*S. mutans* activity named salivaricin M.

Using the sequenced genome, the nucleotide sequence for the complete 16S rRNA gene for *S. salivarius* M18 was compared to the database of DNA sequences held at the National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (BLAST) program. The closest matches for the DNA sequence were to *S. salivarius* and *S. thermophilus*. Specifically, comparison of the 1,537 base pairs of the *S. salivarius* M18 sequence indicated there were minimal genetic differences compared to *S. salivarius* (99.8% homologous) and *S. thermophilus* (99.61% homologous). For confirmation of the species identity, the whole genome was also analyzed using KmerFinder 2.0, a bioinformatics tool hosted by the Center for Genomic Epidemiology[1]. KmerFinder predicts prokaryotic species on the basis of similarity between overlapping kmers (substrings of k nucleotides in DNA sequence data) of a query genome and those within a validated whole genome database (Hasman *et al*., 2014; Larsen *et al*., 2014). Kmer analyses of whole genome data represents a third-generation sequencing technology that has higher resolution and more phylogenetically accurate classifications than that provided by traditional 16S rRNA analysis methods (Larsen *et al*., 2014). Analysis of the whole genome of *S. salivarius* M18 using KmerFinder against 16-mer sequences generated from 1,647 complete bacterial genomes downloaded from the NCBI database identified *S. salivarius* CCHSS3 and JIM8777 as species matches confirming the identity of the M18 strain as *S. salivarius* (Table 2.1.5.1-1).

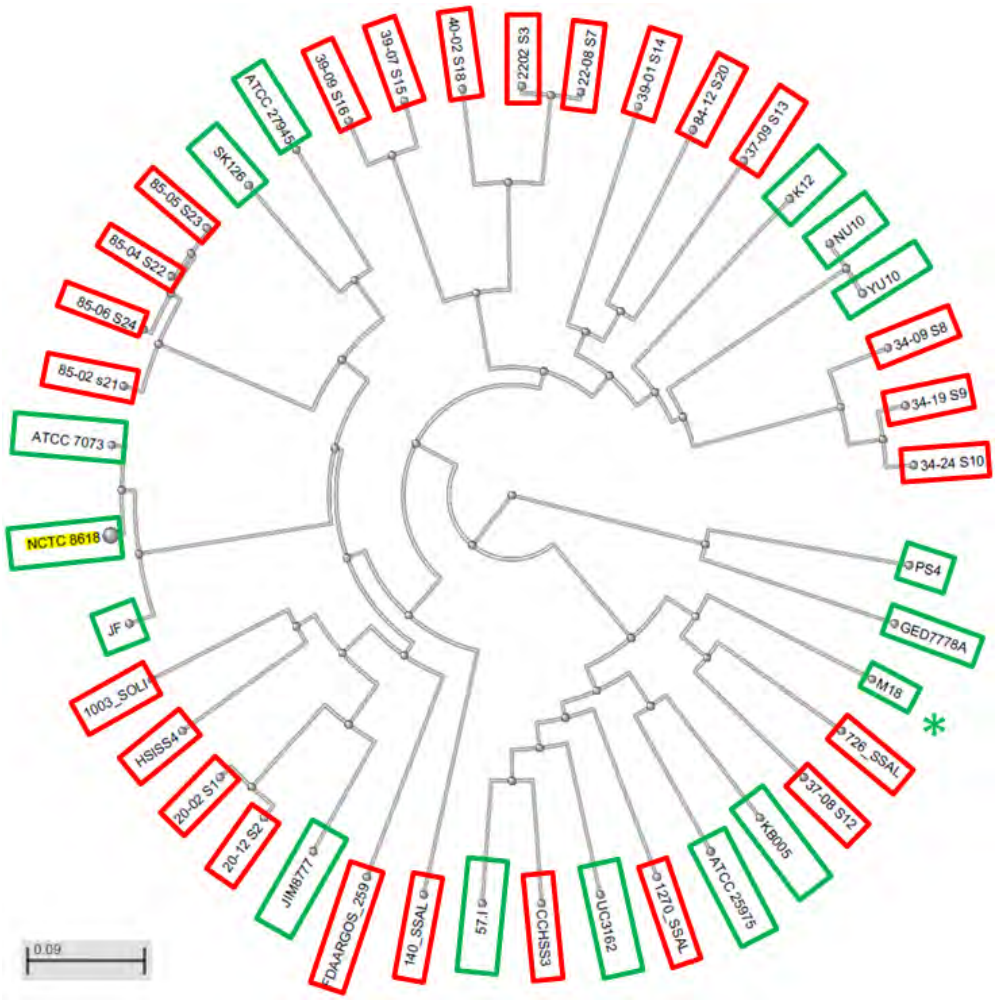
Table 2.1.5.1-1 Species Identity Verification Using KmerFinder 2.0

Template Alignment	Z-score	P Value	Query Coverage (%)	Template Coverage (%)	Depth	Total Query Coverage (%)	Total Template Coverage (%)
Streptococcus salivarius, Streptococcus salivarius CCHSS3	3395	776.7	63.73	66.65	0.67	63.73	66.65
Streptococcus salivarius, Streptococcus salivarius JIM8777	499	223.5	9.37	9.83	0.10	51.38	53.94

Whole genome BLAST comparisons of available sequenced *S. salivarius* genomes are shown below in Figure 2.1.5.1-1. The *S. salivarius* M18 genome clustered in a separate group from *S. salivarius* K12. Strains isolated from healthy subjects and clinical isolates in subjects with iatrogenic cases of sepsis did not cluster within any specific group; a finding that is consistent with findings by Delorme and colleagues who reported that commensal and infection-associated *S. salivarius* strains could not be distinguished by cluster analyses (Delorme *et al*., 2007, 2015). These findings strongly support the conclusion that clusters of *salivarius* strains with pathogenic or unique opportunistic phenotypes do not exist for the species.

[1] https://cge.cbs.dtu.dk/services/KmerFinder/

Figure 2.1.5.1-1 Dendrogram of *Streptococcus salivarius* Genome BLAST



Data represents BLAST comparisons of *S. salivarius* whole genome sequences deposited in the NCBI GenBank database using NCTC 8618 as a reference template. Isolates from iatrogenic sepsis related cases are displayed in red boxes and samples obtained from healthy individuals displayed in green. Strain NCTC 8618 is the type strain for the species.

2.1.5.2 Genetic Similarity to the Yogurt Starter Streptococcus thermophilus

Comprehensive discussions of the genetic relationship with *S. salivarius* and *S. thermophilus* are discussed in Section II.D.2 of GRN 591 (U.S. FDA, 2016). In brief, *S. thermophilus* was considered a distinct species; however, the taxonomic status of the organism had been in question for several years, and some investigators proposed that *S. thermophilus* is, in fact, a subspecies of *S. salivarius*. This taxonomic discrepancy first appeared in the literature in 1984 in studies based on the work of Farrow and Collins (1984). Using DNA base composition, DNA-DNA homology, and long-chain fatty acid analyses, the authors determined that *S. thermophilus* and *S. salivarius* possess similar mol % G + C values (about 37 to 41), long-chain fatty acid profiles, and belonged to a single DNA homology group. Based on this information and earlier studies, the authors proposed that *S. thermophilus* be reclassified as *S. salivarius* subsp. *thermophilus* comb. nov. (Farrow and Collins, 1984); however, results of subsequent DNA-DNA re-association experiments under more stringent conditions indicated that *S. thermophilus* was most likely a distinct species (Schleifer *et al.*, 1991). Based on their findings, Schleifer *et al.* (1991) suggested that the name

should be shifted back to its former one, though it is still widely reported as *S. salivarius* ssp. *thermophilus* (Bylund, 1995)[2].

Although the contention on whether *S. salivarius* and *S. thermophilus* are taxonomically identical species has been debated for a number of years, recent phylogenetic comparisons of the Salivarius-group streptococci suggests that divergence of *S. salivarius* occurred early in the evolution of the salivarius lineage, and that *S. thermophilus* and *S. vestibularis* are more closely related organisms; these observations imply that members of *S. thermophilus* and *S. salivarius* should be regarded as separate species. This distinction also is consistent with the well-established differences between the ecological niches for which each species inhabits; *S. thermophilus* adapted to a milk-based environment compared to *S. salivarius* being adapted to the oral cavity. Nevertheless, the close genetic relationship between *S. salivarius* and *S. thermophilus*, and the long-history of safe use of *S. thermophilus* in yogurt starters strongly supports the contention that the evolution of pathogenic traits has not occurred in this lineage.

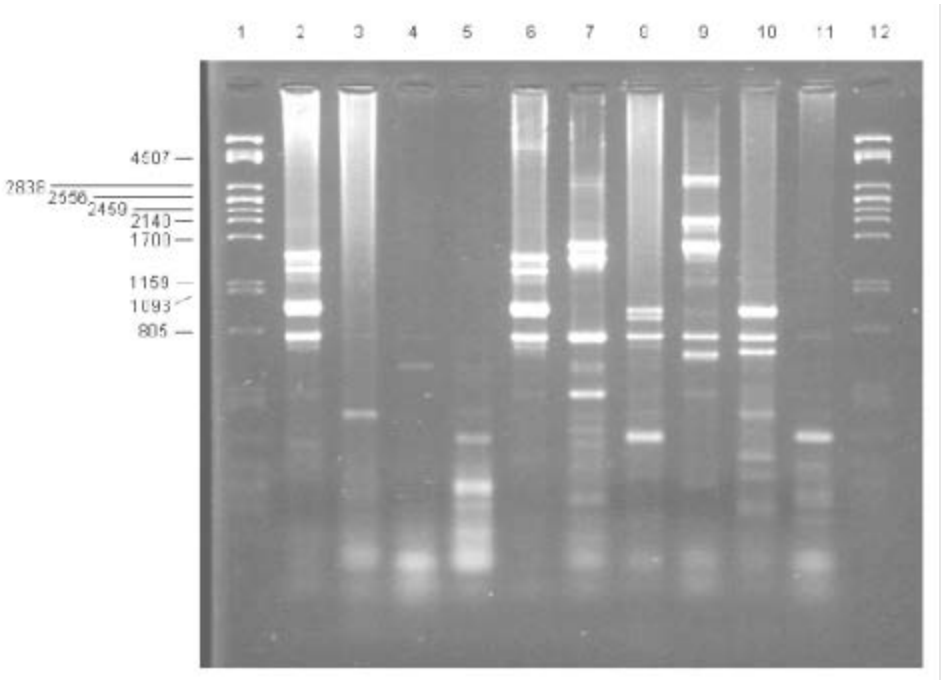
2.1.5.3 Strain Characterization

2.1.5.3.1 ERIC-PCR Profile of *S. salivarius* M18

Using Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR molecular typing, a strain-specific genomic fingerprinting method capable of resolving *S. salivarius* M18 at the strain level was developed. As shown in Figure 2.1.5.3.1-1, the ERIC-PCR fingerprint generated a gel electrophoresis fragment pattern that was specific to *S. salivarius* M18. The relationship between *S. salivarius* strains was investigated using the image analysis software Quantity One system (BioRad). The dice coefficient was calculated by comparing all bands with no weighting, and the phylogenetic relationship was determined using neighbor-joining. As shown in Figures 2.1.5.3.1-1 and 2.1.5.3.1-2, the ERIC-PCR profiles and neighbor-joining demonstrate that *S. salivarius* M18 (Lane 10) can be distinguished from other *S. salivarius* strains (Lanes 2 to 9; 11), including *S. salivarius* K12 (Lane 9).

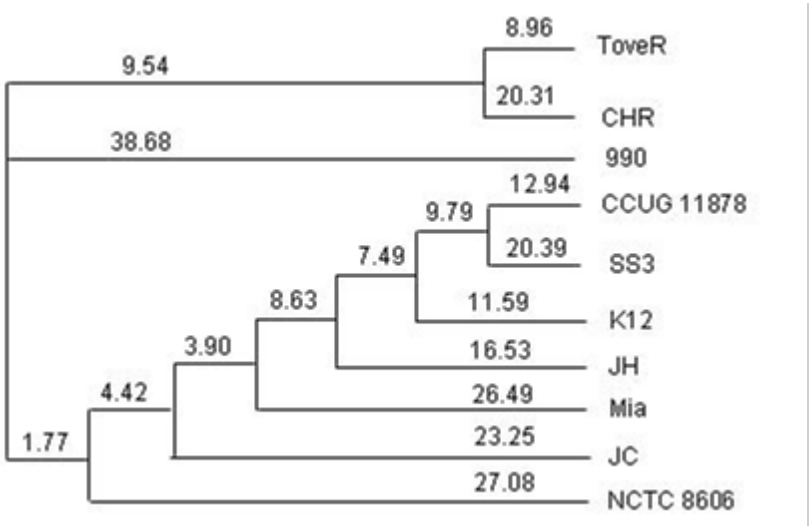
[2] Chapter 10 of the Dairy Processing Handbook. Gösta Bylund (Ed.). 1995; Tetra Pak Processing Systems AB, S-221 86 Lund, Sweden which derives from an International Dairy Federation Bulletin (IDF 263) now classifies *S. thermophilus* as *S. salivarius* ssp. *thermophilus*; http://www.dairykorea.com/fdk/2000/2368/2368-00235.htm

Figure 2.1.5.3.1-1 ERIC-PCR Profile of Various *Streptococcus salivarius* Strains



Lanes 1 & 12 DNA molecular weight marker (Pst I digest of lambda DNA); lanes 2-11 *S. salivarius* strains, CCUG 11878, 990, NCTC 8606, CHR, SS3, JC, JH, K12, M18, and ToveR, respectively.

Figure 2.1.5.3.1-2 Phylogenetic Relationship Between *Streptococcus salivarius* Strains Determined by Neighbor-Joining Analysis of ERIC-PCR Profiles

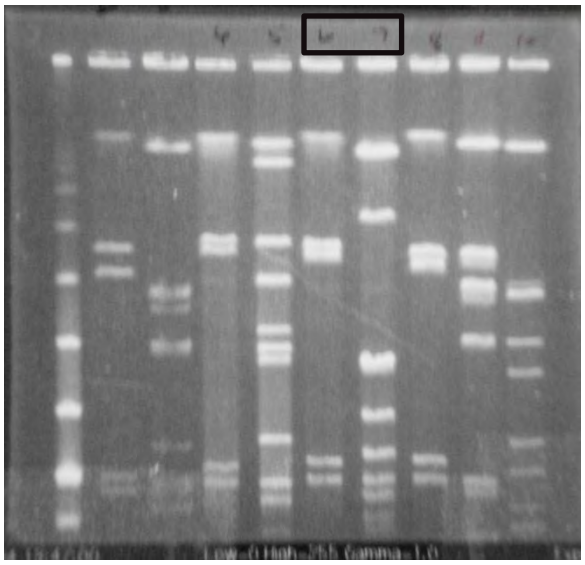


Numbers represent the branch lengths. Mia = *S. salivarius* M18.

2.1.5.3.2 PFGE Profile of *S. salivarius* M18

Pulsed Field Gel Electrophoresis (PFGE) of restricted chromosomal DNA is the current 'gold standard' for bacterial strain differentiation. The unique *Sma*I and *Iceu*I-PFGE profiles of *S. salivarius* M18 were determined and compared with other *S. salivarius* strains (see Figure 2.1.5.3.2-1; Lanes 6 and 7). Similar to the ERIC profiling, this genetic technique also demonstrates that *S. salivarius* M18 can be differentiated from other commercial as well as naturally occurring *S. salivarius* strains.

Figure 2.1.5.3.2-1 PFGE Analysis of *Streptococcus salivarius* M18



Lane: 1- DNA marker, 2 - *S. salivarius* strain NR (*Iceu*I), 3 - NR (*Sma*I), 4 - G39 (*Iceu*I), 5 - G39(*Sma*I), **6 - M18 (*Iceu*I), 7 - M18 (*Sma*I),** 8 - TrevP (*Iceu*I), 9 - TrevP (*Sma*I digest) 10 - K12 (*SmaI*)

2.2 Manufacturing

S. salivarius M18 is produced under current Good Manufacturing Practices (cGMP), using food-grade ingredients and processing aids that have GRAS status for their intended use, or are used in compliance with appropriate federal regulations. Quality control methods are implemented throughout various stages of the fermentation process to ensure production of a pure culture that is devoid of any contaminating pathogens. Master and working cultures are used for assurance of strain stability and consistency of the fermentation process. Sucrose, skim milk powder and ammonia salts are used as carbon and nitrogen sources, and yeast extract is used as source of essential nutrients. *S. salivarius* M18 is manufactured using the same processing-aids and additives and general procedures as those used for *S. salivarius* K12 and therefore, this information is incorporated by reference to Section II.E of GRN 591.

2.3 Product Specifications and Batch Analyses

2.3.1 Proposed Product Specifications

The specifications for *S. salivarius* M18 are presented in Table 2.3.1-1 and include physical, chemical, and microbial parameters.

Table 2.3.1-1 Product Specifications for *Streptococcus salivarius* M18

Specification Parameter	Specification	Method
Active		
S. salivarius (CFU/g)	NLT $1.0x10^{11}$	BLIS Technologies Ltd SOP PO11
Microbial		
Coliforms (CFU/g)	Not detectable	Compendium 4th Edition 2001 (mod)
Escherichia coli (CFU/g)	Not detectable	Compendium 4th Edition 2001 (mod)
Salmonella spp. (CFU/25 g)	Not detectable	ISO 6579:2002 (E)
Mesophilic aerobic spores (CFU/g)	NMT 200	Compendium 4th Edition 2001
Staphylococcus aureus (CFU/g)	Not detectable	ISO 6888-3:2003
Molds (CFU/g)	NMT 50	British Pharmacopeia, 2010
Yeast (CFU/g)	NMT 50	British Pharmacopeia, 2010
Physical Characteristics		
Water activity (a_w)	<0.25	AquaLab Operator's Manual
Particle size	$_d(0.9)$ <500 µm	Mastersizer 2000[a]
Heavy Metals		
Arsenic	NMT 1 ppm	ICP-MS (APHA3125B)
Lead	NMT 0.5 ppm	ICP-MS (APHA3125B)
Mercury	NMT 0.15 ppm	ICP-MS (APHA3125B)
Cadmium	NMT 0.2 ppm	ICP-MS (APHA3125B)

APHA = American Public Health Association; CFU = colony-forming units; ICP-MS = inductively coupled plasma mass spectrometry; ISO = International Organization for Standardization; NLT = not less than; NMT = not more than; ppm = parts per million;
[a] Manufacturers internal test.

2.3.2 Batch Analyses

Analysis of 3 non-consecutive lots of *S. salivarius* M18 demonstrates that the manufacturing process as described in Section 2.2 produces a consistent product that meets specifications. A summary of the batch analyses for the 3 lots of *S. salivarius* M18 is presented in Table 2.3.2-1 (see Appendix A for the corresponding Certificates of Analysis).

Table 2.3.2-1 Product Analysis for 3 Non-Consecutive Lots of *Streptococcus salivarius* M18

Specification Parameter	Specification	Manufacturing Lot		
		13.02	16.06	16.10
Active				
S. salivarius (CFU/g)	NLT $1.0x10^{11}$	$>1x10^{11}$	$>1x10^{11}$	$>1x10^{11}$
Microbial				
Coliforms (CFU/g)	Not detectable	ND	ND	ND
Escherichia coli (CFU/g)	Not detectable	ND	ND	ND
Salmonella spp. (CFU/25 g)	Not detectable	ND	ND	ND
Mesophilic aerobic spores (CFU/g)	NMT 200	<10	<10	<10
Staphylococcus aureus (CFU/g)	Not detectable	ND	ND	ND
Molds (CFU/g)	NMT 50	<10	<10	<10
Yeast (CFU/g)	NMT 50	<10	<10	<10
Physical Characteristics				
Water activity (a_w)	<0.25	<0.2	<0.2	<0.2
Particle size	$_d$(0.9) <500 µm	$_d$(0.9): 373 µm	$_d$(0.9): 397 µm	$_d$(0.9): 367 µm
Heavy Metals				
Arsenic	NMT 1 ppm	<0.05	<0.05	<0.05
Lead	NMT 0.5 ppm	<0.05	<0.05	<0.05
Mercury	NMT 0.15 ppm	<0.05	<0.05	<0.05
Cadmium	NMT 0.2 ppm	<0.02	<0.02	<0.02

CFU = colony-forming units; ND = not detected; NLT = not less than; NMT = not more than; ppm = parts per million.

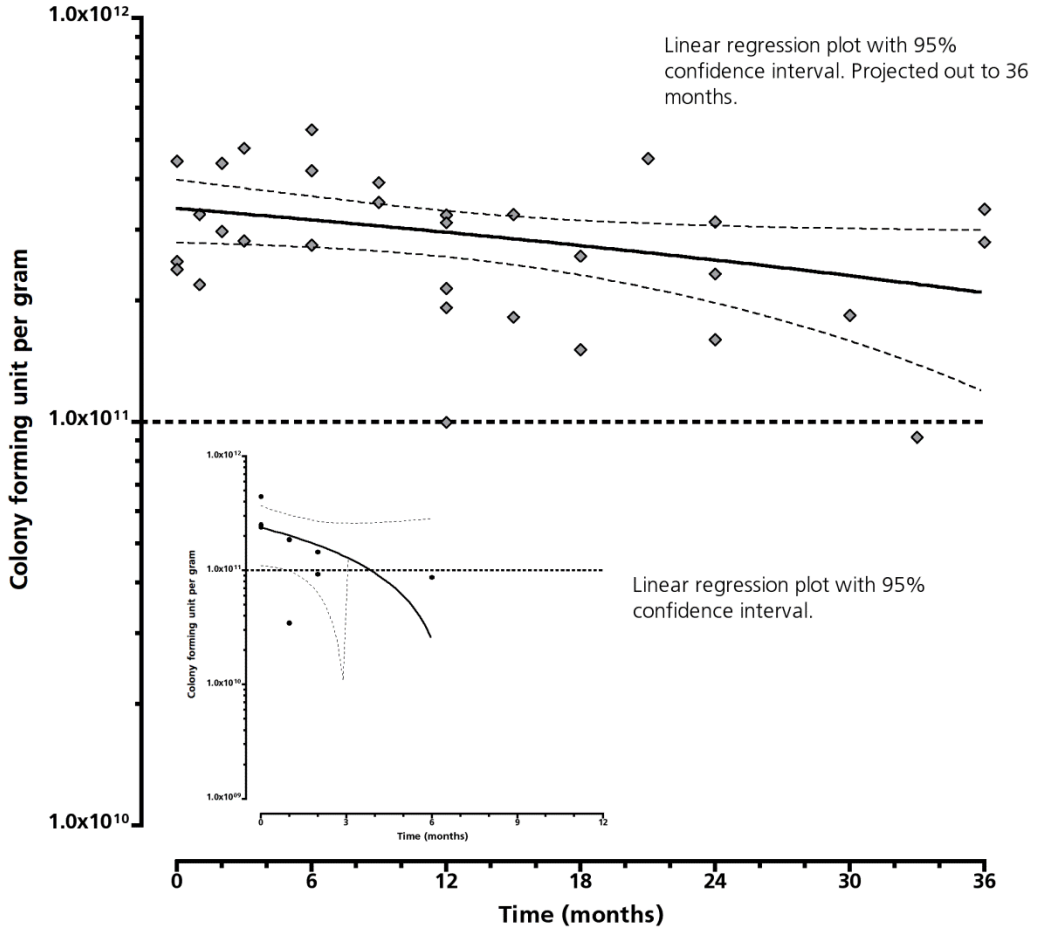
2.4 Stability

2.4.1 Storage Stability

The stability of freeze-dried *S. salivarius* M18 powder was assessed at storage temperatures between 2°C and 8°C. Cell counts were conducted over a period of 36 months, and as shown in Figure 2.4.1-1, the material was stable at 5±3°C with only 1 data point falling below the $1x10^{11}$ CFU per gram ingredient specification cut-off. This data point (at 33 months) appears to be an anomaly, as the 36-month count for that batch was well above the specification cut-off. Best fit modelling of the data yields a linear regression line that clearly forecasts stability beyond 36 months (2-fold above the specification cut-off). BLIS has an on-going program to monitor batch stability and is confident that when packaged and stored correctly, BLIS's *S. salivarius* M18 ingredient is stable at 5±3°C for up to at least 36 months.

The insert plot (lower left) shows the fate of the same material if stored at 37°C/75% relative humidity, in this case the $1x10^{11}$ CFU per gram line is rapidly breached (approximately 4 months) in what appears to be a linear decay, emphasizing the need to adhere to the storage conditions specified for the product.

Figure 2.4.1-1 Stability of *Streptococcus salivarius* M18 Lyophilized Commercial Product Stored at 5±3°C for 36 Months and 37°C/75% Relative Humidity (insert)



Part 3. §170.235 Dietary Exposure

3.1 History of Use in Food

Strains of *S. salivarius* have a long history of use as a starter culture for the manufacture of cheese and yogurt; however, the species is no longer widely used in the manufacture of food products in the North American market, as the genetically-related strain *S. thermophilus* has proven to be a superior species for uses in yogurt starter cultures. *S. salivarius* also has been detected in traditional fermented milks (Abdelgadir *et al*., 2001), as well as raw milk Salers cheese and Serbian craft cheeses (Callon *et al*., 2004; Pešić-Mikulec and Jovanović, 2005). The species *S. salivarius* also is listed on the International Dairy Federation list of Microbial Fermentation Cultures with technological beneficial use (Bourdichon *et al*., 2012). In the U.S., the strain *S. salivarius* K12 has GRAS status for use in a variety of conventional food and beverage products including: baby, infant, and toddler foods (excluding infant formula); baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices;

soft candy; sweet sauces, toppings, and syrups; at levels up to 20 mg per serving (providing a minimum of $1x10^9$ CFU per serving) (U.S., FDA, 2017).

3.2 Estimated Consumption of *S. salivarius* M18 from All Intended Conditions of Use in Food

The estimated intake of *S. salivarius* from the intended food-uses described in Table 1.3-1 has been previously reported as part of the GRAS evaluation for *S. salivarius* K12 (see Section IV.A.2 of GRN 591). Since the food uses and use levels of *S. salivarius* M18 described in Section 1.3 of this notice are identical to those described for *S. salivarius* K12 in GRN 591, the daily intake estimates previously derived for *S. salivarius* K12 can be extrapolated to food uses of *S. salivarius* M18. The estimated intakes for *S. salivarius* M18 described in Table 3.2-1 below has been prepared using data presented in GRN 591 (U.S. FDA, 2016). It is expected that food uses of *S. salivarius* M18 would generally be substitutional to food uses of *S. salivarius* K12; however, as M18 is not intended to serve as a replacement for K12, some additive consumption may occur on occasion. Given the logarithmic nature of microorganism counts, even a doubling of the intake estimates described below in Table 3.2-1 would remain with the 10^{10} CFU count range. BLIS has therefore concluded that any additive exposures would not appreciably affect the intake estimates. As discussed in GRN 591, interpretation of the intake estimates presented in Table 3.2-1 below should consider that the intake methodology used is generally considered to be 'worst case' because of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently.

Table 3.2-1 Summary of the Estimated Daily Intake of *Streptococcus salivarius* M18 per CFU from Proposed Food-Uses in the U.S. by Population Group (2003-2004, 2005-2006 NHANES Data)

Population Group	Age (Years)	Percent Users	Actual # of Users	All Person (CFU)		All User (CFU)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	90.0	1,722	$9.2x10^9$	$1.6x10^{10}$	$1.0x10^{10}$	$1.7x10^{10}$
Children	3 to 11	99.8	2,728	$1.1x10^{10}$	$1.8x10^{10}$	$1.1x10^{10}$	$1.8x10^{10}$
Female Teenagers	12 to 19	98.8	1,964	$9.6x10^9$	$1.8x10^{10}$	$9.7x10^9$	$1.8x10^{10}$
Male Teenagers	12 to 19	98.1	1,903	$1.2x10^{10}$	$2.3x10^{10}$	$1.2x10^{10}$	$2.3x10^{10}$
Female Adults	20 and up	97.3	4,164	$8.3x10^9$	$1.7x10^{10}$	$8.6x10^9$	$1.7x10^{10}$
Male Adults	20 and up	96.1	3,692	$9.8x10^9$	$2.0x10^{10}$	$1.0x10^{10}$	$2.1x10^{10}$
Total Population	All ages	96.9	16,173	$9.5x10^9$	$1.9x10^{10}$	$9.8x10^9$	$1.9x10^{10}$

CFU = colony-forming units; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the notified ingredient.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

Streptococcus salivarius is a dominant species of the oral mucosa of all humans, and viable cultures have been isolated from saliva, human milk, intestinal samples, and fecal samples (see Section 6.2 below). The species is genotypically indistinguishable from the safe industrial microorganism *S. thermophilus*, a species that has a long history of use in dairy fermentation. *S. salivarius*, although less commonly used in food fermentation is listed on the International Dairy Federation inventory of microorganisms with technological beneficial use in food fermentations (Mogensen *et al*., 2002; Bourdichon *et al*., 2012). Consistent with this established history of safe use, the strain *S. salivarius* K12 has GRAS status for use in a variety of food and beverage products at use-levels of up to $2x10^9$ CFU per serving (GRN 591 – U.S. FDA, 2016). *S. salivarius* K12 also has been cleared by Health Canada and the Australia New Zealand Food Safety Authority (FSANZ) and is a Listed Medicine by the Australian Therapeutics Goods Administration (TGA). *S. salivarius* M18 has been cleared by FSANZ (Appendix B) for general food use based on the history of use of the species in food and corresponding exemption from the novel food regulations in Australia/New Zealand. Accordingly, the totality of data and information supporting the safety of the species for use in food has been the subject of multiple systematic and comprehensive reviews by qualified experts. Generally available discussions on the history of use, non-pathogenicity, and non-toxicogenicity of the species are incorporated by reference to GRN 591 (U.S. FDA, 2016) and are discussed in brief in their respective sections below as they apply to *S. salivarius* M18.

To identify additional scientific publications relevant to the safety of *S. salivarius* M18, comprehensive and detailed searches of the published literature were conducted using the electronic databases Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. It has been noted that the species has been identified in various cases of iatrogenic infections; however, these cases have been concluded to be opportunistic in nature and the available evidence strongly supports a conclusion that the opportunistic nature of the species is no greater than that of other probiotic organisms. Updated searches of the literature did not identify case reports to suggest that pathogenic strains exist within the species (see Section 6.5.1). The complete genome of *S. salivarius* M18 has been sequenced and is publicly available on the GenBank database (Heng *et al*., 2011). Several bioinformatic analyses of the whole genome have been conducted to identify genes encoding undesirable factors (*e.g.*, Streptococcal toxins, virulence determinants, and antibiotic resistance genes), or genes suggestive that the strain may have virulence phenotypes. These analyses included BLAST analyses of the whole genome and plasmid for identification of gene products corresponding to putative and established virulence factors against genes hosted in multiple curated and up-to-date databases (*e.g.*, PATRIC-VF, VFDB, Victors, CARD), as well as predictive modelling of the whole genome for pathogenic potential using validated methods described by Cosentino *et al*. (2013). Results of the bioinformatics evaluation are presented in Section 6.5.2 below; no novel gene products expected to impart undesirable phenotypes to the strain were identified. It was therefore concluded that *S. salivarius* M18 is a non-pathogenic and non-toxicogenic strain. This conclusion is corroborated by published human investigations evaluating the oral

consumption of *S. salivarius* M18. These studies are discussed in Section 6.4 below. Administration of up to $7.2x10^9$ CFU per day for 90 days was well tolerated in healthy children. These levels are consistent with anticipated intakes of *S. salivarius* M18 occurring from the intended food-uses described in Section 1.3.

Based on published data and information described above, a safety assessment of *S. salivarius* M18 was conducted using the decision tree described by Pariza *et al*., for the safety assessment of microbial cultures for consumption by humans and animals (Pariza *et al*., 2015). The findings of the decision tree analysis are presented in Section 6.8, and it was concluded that *S. salivarius* M18 was appropriate for human consumption.

6.2 Colonization and Metabolic Fate

S. salivarius predominantly inhabits the dorsum of the tongue and the pharyngeal mucosa in humans (Gibbons and van Houte, 1975). It becomes established in the human oral cavity within 2 days after birth. The levels of *S. salivarius* in swab samples taken from new-born infants represent 10% of the total streptococci isolated, increasing to 25% to 30% by 1 month of age (Hedge and Munshi, 1998). In adults, *S. salivarius* represent 17% of the total streptococci isolated from the tongue and 30% from the pharynx (Frandsen *et al.,* 1991). In non-stimulated saliva samples taken from children and adults, the population levels of *S. salivarius* range from 10^7 to 10^8 CFU per mL. The total saliva volume produced per day is approximately 500 mL for children (Watanabe *et al.,* 1995) and up to 1.5 L for adults (Porter *et al.,* 2004; Wescombe *et al.,* 2006); therefore, the daily consumption of commensal *S. salivarius* in humans is estimated to range from $5x10^9$ to $1.5x10^{11}$ CFU/day.

Commensal *Staphylococcus* and *Streptococcus* sp. have been reported to be one of many predominant bacterial species in breast milk, and the identification of *S. salivarius* in breast milk has been reported by several investigators (Heikkilä and Saris, 2003; Martín *et al.,* 2004, 2007; Dalidowitz, 2005). Consistent with the common presence of *S. salivarius* in the oral cavity and in human breast milk, *S. salivarius* isolates have been reported in fecal samples of infants within the first 3 days and were a predominant species throughout the breastfeeding period (Favier *et al.*, 2002; Park *et al.*, 2005). Viridans streptococci have been reported to antagonize oral colonization by methicillin-resistance *S. aureus* in infants, and similar effects on *S. aureus* growth has been reported with *S. salivarius* isolates obtained from breast milk. Kirjavainen *et al.* (2001) reported that the presence of viridian group streptococci was a common feature of the healthy infant gut and in contrast to that observed among atopic infants. The widespread presence and early colonization of *S. salivarius* in the oral cavity, and gastrointestinal tract of infants, its presence in human breast milk, and preliminary findings that the members of *S. salivarius* can competitively displace/inhibit the growth of pathogenic microorganisms, suggests that the species may play an important and unappreciated nutritional role in human biology, and requires further investigation.

It is well established that *Streptococcus* sp. form a dominant phylum throughout all gastrointestinal sites proximal to the terminal ileum (Wang *et al*., 2005; Booijink *et al*., 2007; Booijink *et al*., 2010; Zoetendal *et al*., 2012; Sundin *et al*., 2017), and *S. salivarius* isolates are routinely identified as a major streptococcal species within these sites. Hakalehto *et al*., (2011) identified isolates of *S. salivarius* from human stomach biopsy samples at levels suggestive of low-level colonization, and Van den Bogert *et al*., (2013a) identified cultivatable isolates of *S. salivarius* from human small intestinal samples. In the study by Van den Bogert *et al*., (2013) the authors obtained small intestinal effluent samples from 6 ileostomy subjects and small-intestinal fluid samples from 6 healthy subjects. Fecal samples from 10 individuals also were investigated. Intestinal samples were cultivated in Mitis Salivarius agar and isolates selected using DNA fingerprinting followed by whole genome sequencing. The authors reported that gene sequencing results of 34 intestinal and 10 fecal samples collected from 19 individuals revealed that at least two *S. salivarius* lineages were

present in almost all small-intestinal samples and in several fecal samples indicating that these strains are "*common colonizers and represent an important population of, in particular, the small-intestinal microbiota*" (Van den Bogert *et al*., 2013a). Regions of the gastrointestinal tract proximal to the terminal ileum are environments where food components encounter bacteria. *Streptococci* sp. exhibit very fast sugar transport and metabolism systems and are therefore well adapted for survival in this environment where uptake of nutrients and gastrointestinal transit is rapid (Booijink *et al.*, 2010; Zoetendal *et al*., 2012). The rapid internalization and conversion of simple carbohydrates to support growth is a prominent strategy for microbial proliferation and microbiota maintenance in the small intestine and differentiates the microbiota of the small intestine with populations residing within the large intestine, which is largely absent of *Streptococcus* sp. (Wang *et al*., 2005; Sundin *et al*., 2017). The functional significance of microbiota populations within the small intestine are unclear; however, studies conducted using germ-free animals have suggested that microbial populations indigenous to the small intestine can influence host gene expression including upregulation of nutrient transport genes, and upregulation of genes with putative roles in mucosal barrier function and epithelial barrier integrity (Leser and Mølbak, 2009). The metabolism of streptococcal populations of the small intestine appears to be closely inter-dependent with that of indigenous *Veillonella* and this represents a prominent and important role especially of *S. salivarius* populations in host nutrition and in the maintenance of a healthy and stable intestinal microbiota (Zoetendal *et al*., 2012; van den Bogert *et al*., 2013b).

6.2.1 Effects on Oral Microbiota

Burton *et al.* (2013a) evaluated the oral colonization of *S. salivarius* M18 in a population of young adults (average age of 19 years; gender not reported) that consumed lozenges containing *S. salivarius* M18 (CFU per day: $1x10^6$, n=19; $1x10^7$, n=20, $1x10^8$, n=17, $1x10^9$, n=19) for 28 days. Saliva samples were collected at baseline and weekly thereafter to measure salivary *S. salivarius* M18 for each subject. Total mean salivary *S. salivarius* did not change from baseline following exposure to *S. salivarius* M18 for 28 days, indicating that exposure to *S. salivarius* M18 did not disrupt the indigenous oral microflora. On the other hand, *S. salivarius* M18 numbers increased with the dose quantity during the first week (Day 7), suggesting that a proportion of the original *S. salivarius* population was replaced by strain M18. *S. salivarius* M18 remained elevated for the remainder of the study with a slight downward trend from Week 1. The percentage of subjects with detectable levels of *S. salivarius* M18 in their saliva also increased with the dose quantity, with the lowest dose group having a maximum of about 42% of subjects with *S. salivarius* M18 at Day 14, for example, whereas 100% of subjects had *S. salivarius* M18 in the highest dose group at Days 7 and 14. Levels of *S. salivarius* M18 were not measured after lozenge consumption ceased.

Burton *et al.* (2013b) evaluated whether dosing with *S. salivarius* M18 resulted in persistent colonization of the oral cavity in a group of healthy male and female school children (average age 8.9 years) with a history of dental caries. Children in the probiotic group consumed 2 lozenges per day each containing $3.6x10^9$ CFU *S. salivarius* M18 (n=40) for 3 months and the placebo group (n=43) received lozenges without probiotic. Despite a high rate of compliance throughout the study (>80%) following 3 months of probiotic consumption only 22% of subjects had detectable levels of *S. salivarius* M18 in their saliva. Specifically, at the end of the treatment period the salivary populations of *S. salivarius* M18 in 9 of the 40 probiotic subjects were at least 5% of the total populations of *S. salivarius*. Overall, plaque scores were significantly lower in the probiotic group compared to control levels, particularly for this subgroup of subjects who did colonize with *S. salivarius* M18 and this appeared to be associated with decreased levels of salivary *S. mutans.*

Findings discussed above demonstrate that at high doses *S. salivarius* M18 can colonize the oral cavity of consumers, and that this colonization is associated with oral health benefits. As such, the consumption of *S. salivarius* M18 from intended food-uses may colonize the oral cavity but will not likely induce permanent/significant changes in the overall oral microflora composition, particularly given that consumption of indigenous strains of *S. salivarius* within saliva occurs on a continual basis in all individuals.

6.2.2 Effects on Gastrointestinal Microbiota

There is no strain-specific information characterizing the gastrointestinal colonization of *S. salivarius* M18; however, the indigenous microflora profiles of most animals are intrinsically highly stable and resistant to colonization by exogenous microorganisms. Permanent lifelong colonization by ingested microorganisms is rare (WHO/FAO, 2009).

The capacity of *S. salivarius* K12 to survive and colonize the rodent gastrointestinal tract was evaluated by Lee *et al*. (2009). Six-month-old male Wistar rats were provided gavage doses of a microorganism mixture containing *Lactobacillus acidophilus* LA741 ($3.9x10^9$ CFU), *Lactobacillus rhamnosus* L2H ($2.3x10^{10}$ CFU), *Bifidobacterium lactis* HN019 ($8.0x10^9$ CFU), and *S. salivarius* K12 ($1.1x10^{10}$ CFU) twice daily for 3 days. Intestinal contents, mucus, and feces were evaluated for microbial colonization of the administered strains at 6 hours, 3 days, and 7 days after the last gavage dose using Denaturing Gradient Gel Electrophoresis (DGGE), and culturing in selective media. At 6 hours, viable cells were detected for all 4 strains within samples obtained from the feces, the lumen contents and mucous layers of the ileum and colon. However, by Days 3 and 7, no viable cells or DGGE DNA banding corresponding to *S. salivarius* K12 were detected in any of the samples, and the authors concluded that "*S. salivarius* DNA *is rapidly released and destroyed when the cells enter the rat GIT* [gastrointestinal tract]". As discussed in GRN 591 the species *S. salivarius* is specific to humans and therefore findings in rodent studies are of unclear relevance to the *in vivo* situation in humans.

Consumption of *S. salivarius* M18 in the diet is not expected to affect the microbiota composition of the gut, particularly given that consumption of indigenous strains of *S. salivarius* within saliva occurs in all individuals on a continual basis. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the cellular components (proteins, lipids, carbohydrates) used as a source of nutrients. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the feces.

6.3 Toxicology Studies

No strain specific studies examining the potential toxicological effects of *S. salivarius* M18 have been reported. Rodent toxicity information has been reported for *S. salivarius* in GRN 591. All toxicological studies conducted to date with *S. salivarius* strain K12 that were previously presented in GRN 591 are summarized below. An updated search of the scientific literature up to 28 February 2018 did not identify any new toxicological studies with this strain. None of the studies conducted with *S. salivarius* K12 reported any toxicological findings or other biological effects that warrants special considerations for other strains within the species. As discussed in Section IV of GRN 591 "*Microorganism-host interactions are species specific. The species S. salivarius is unique to humans, and toxicity studies conducted using rodents or other animal species administered S. salivarius at high dietary concentrations are expected to be of limited relevance to humans (ILSI, 1995)*". Findings from available toxicity studies of *S. salivarius* K12 were therefore considered corroborative in nature.

6.3.1 Toxicity Studies on *S. salivarius* K12

6.3.1.1 Acute Toxicity Studies

The acute oral toxicity of the freeze-dried *S. salivarius* K12 powder (*i.e.,* combination of *S. salivarius* and a maltodextrose sugar lyoprotectant) was investigated in Sprague-Dawley rats as a preliminary step in evaluating dose ranges for a repeated-dose toxicity study (Burton *et al.*, 2010). This study was conducted in accordance with the Organisation of Economic Co-operation and Development (OECD) Guideline No. 407 (OECD, 2008). Fifty-nine rats were randomized into 5 groups. Groups 1, 2, and 3 (6/sex/group) were administered a bolus dose of the freeze-dried *S. salivarius* K12 powder providing either $1.25x10^8$, $1.67x10^9$, or $8x10^{10}$ CFU per rat (equivalent to 7.5, 100, and 5,000 mg/kg body weight, respectively). The remaining 2 groups were administered the lyoprotectant (equivalent to the lowest dose of *S. salivarius* K12; n=6/sex) or sterile saline (n=3/sex). An additional male rat was included in each group and was euthanized 48 hours after administration and evaluated for septicemia or acute bacterial infections of the heart valves and pharyngeal tissues. The remaining animals were observed twice daily for 14 days for clinical signs, mortality, and food consumption. Upon completion of the observation period, all animals were necropsied, and biochemical and hematological evaluations were conducted. Four animals in each group were further examined for gross abnormalities. Oral administration of *S. salivarius* K12 was reported to have no effects on food consumption and there were no signs of tissue abnormalities. The authors concluded that the freeze-dried *S. salivarius* K12 powder was not acutely toxic at doses up to 5,000 mg/kg body weight/day ($8x10^{10}$ CFU/day), the highest dose tested, when orally administered to rats.

6.3.1.2 Repeated-Dose Studies

The repeated-dose toxicity of *S. salivarius* K12 was investigated in mature (<500 g) Sprague-Dawley rats (20/sex/group) fed diets providing 7.5 (low-dose), 100 (mid-dose), or 5,000 mg/kg body weight/day (high-dose) of freeze-dried *S. salivarius* K12 powder for a period of 28 days (Burton *et al.*, 2010; GRN 591 – U.S. FDA, 2016). An additional group of rats was fed a diet providing 7.5 mg lyoprotectant/kg body weight/day. No adverse effects on general clinical signs, ophthalmologic evaluations, organ weights, or gross pathology were reported in any dose group during the administration period. Increased body weights were reported in high-dose males compared to the other male groups (statistics not reported); however, the investigators did not consider this to be biologically relevant since by the end of the 28-day treatment period, the other groups had relatively similar body weights. Increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels were reported in all groups after a 28-day recovery period (Day 56), including the lyoprotectant group (statistics not reported). All other serum biochemistry parameters were reported to be within reference or baseline ranges. Urinalysis and hematology measures did not reveal any significant adverse effects or variations.

Histopathological findings were not considered to be treatment-related as they also occurred in the lyoprotectant or baseline groups or were not evident in any other animal. Inflammatory cell accumulation was reported in the heart and kidneys of 1 animal in each of the treatment groups and these findings were reported to be possibly related to treatment; however, inflammatory cell infiltration was reported in the liver of 2 animals in the lyoprotectant group, and as these histopathological findings are not uncommon in mature rats (<500 g), the reported changes are not considered to be of toxicological concern.

No signs of overt toxicity were reported in mature Sprague-Dawley rats orally administered up to 5,000 mg/kg body weight/day of the freeze-dried *S. salivarius* K12 powder for a period of 28 days; however, a no-observed-adverse-effect level (NOAEL) cannot be determined from this study due to the large number of apparent deviations from OECD and Good Laboratory Practices (GLP) guidelines (OECD, 1998), such as

the use of mature (<500 g) rats, pooling of urinalysis samples, and analyses of primary toxicity endpoints following a 28-day recovery period.

6.3.2 Other Animal Studies

Several studies conducted using mice and rats evaluated the effects of oral administration of *S. salivarius* K12, and other non-related *S. salivarius* strains, on various efficacy related measures. Methodologies used in these studies were not intended to evaluate safety, and findings from these studies were considered to be of limited value to the safety assessment of *S. salivarius* M18. These studies are summarized in Table 6.3.2-1 for completeness. None of the studies presented findings that suggest that the intended use of *S. salivarius* M18 as a food ingredient may present cause for concern.

Table 6.3.2-1 Summary of Other Studies with Various Strains of *Streptococcus salivarius*

Species (Strain, Sex), Number of Animals	Route of Administration and Study Duration	Test Compound	Dose	Reported Effects[a]		Reference
***Streptococcus salivarius* K12**						
Rat (Wistar, male), 5/group	Oral (gavage), twice daily for 3 days	Multi-probiotic: *L. acidophilus* LAFTI L10, *B. lactis* LAFTI B94, and *S. salivarius* K12	2 g probiotic powder (L10 $8x10^8$, B94 $8x10^8$, K12 $2x10^8$ CFU/dose)	Organ and tissue effects	• Probiotic treatment increased the azoreductase activity of the colon contents	Lee *et al.* (2012)
Mice (ICR, female), 7 to 15/group	Oral, 24 & 3 h before, 3, 24, 27 h after *C. albicans* inoculation	*S. salivarius* K12	7.5×10^7, 1.5×10^8 or 3.0×10^8 CFU/dose	Organ and tissue effects	• Histopathology of the tongue indicated reduced pathogenicity of *C. albicans* with probiotic treatment	Ishijima *et al.* (2012)
Mice (CDI, female), 7 to 20/group	Vaginal inoculation, once a day for 3 days or 5 days following group B *streptococcus* (GBS) inoculation	*S. salivarius* K12	1.0×10^8 CFU/dose	Organ and tissue effects	• Inoculation with K12 prevented GBS vaginal colonization	Patras *et al.* (2015)
Other *Streptococcus salivarius* Strains						
Rat (Osborne-Mendel, sex NR) 9 to 10/group	Oral (gavage) Two doses	*S. salivarius* TOVE-R (streptomycin-resistant; colonially rough)	$6x10^8$ CFU/dose	Body weight	• NSD in body weight gain	Tanzer *et al.* (1985)
				Organ and tissue effects	• Initial colonization inhibited the emergence of focally transmitted *S. mutans* 10449 and 6715-13WT.	
Rat (Osborne - Mendel, sex NR) 9 to 13/group	Oral (gavage) 7-8 days following infection with *S. mutans* or *S. sobrinus* Animals were maintained on a high-sucrose diet.	*S. salivarius* TOVE-R (streptomycin-resistant; colonially rough)	$6x10^8$ CFU/day	Body weight	• NSD in body weight gain.	Tanzer *et al.* (1985)
				Organ and tissue effects	• TOVE-R colonized the teeth, but not the tongue • TOVE-R displaced the mutans streptococci on the teeth, which was associated with a significant inhibition of caries induced by *S. mutans* or *S. sobrinus*.	

Table 6.3.2-1 Summary of Other Studies with Various Strains of *Streptococcus salivarius*

Species (Strain, Sex), Number of Animals	Route of Administration and Study Duration	Test Compound	Dose	Reported Effects[a]		Reference
Sprague-Dawley (sex and number NR)	Oral (gavage) 8 doses at 20, 21, 23, 24, 27, 28, 30, and 31 days of age Animals were provided drinking water containing 10^{10} CFU of the bacteria and maintained on a caries-inducing diet.	*S. salivarius* strains HT3R and HT9R	$1X10^{12}$ CFU/day	General condition/ survival	• No adverse effects reported by authors.	Hamada *et al.* (1978)
				Organ and tissue effects	• *S. salivarius* strains were non-cariogenic	

CFU = colony-forming units; NR = not reported; NSD = no significant differences.
[a] Unless stated otherwise, all reported effects are relative to control group(s)

6.3.3 Genotoxicity Studies

As *S. salivarius* M18 was isolated from a saliva sample from a healthy individual, and the species is a dominant organism of the oral mucosa of all humans, it is concluded that mutagenicity and genotoxicity studies are not necessary to conclude that M18 does not represent a mutagenic/genotoxic risk. Furthermore, *S. salivarius* K12 was tested in the Ames assay (up to 5,000 µg/plate) using *Salmonella Typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537, with and without metabolic activation, and was reported to be non-mutagenic (Burton *et al.*, 2010; GRN 591 – U.S. FDA, 2016).

6.4 Human Studies

6.4.1 Studies with *S. salivarius* M18

A randomized double-blind placebo-controlled clinical trial with *S. salivarius* M18 was carried out in healthy male and female school children (average age 8.9 years) with a history of dental caries at the Dental School at the University of Otago, Dunedin, New Zealand (Burton *et al.,* 2013b). Children in the probiotic group consumed 2 lozenges per day each containing $3.6x10^9$ CFU *S. salivarius* M18 (n=40) for 3 months and the placebo group (n=43) received lozenges without probiotic. Saliva samples were collected at baseline, 1, 2, 3, and 7 months for the measurement of indicator microbes (*S. mutans, lactobacilli* and yeast) as well as *S. salivarius* (total count and strain M18). Monthly interviews were conducted with parents/guardians to determine if the children had experienced any adverse events. Colonization of the oral cavity with *S. salivarius* M18 had an antagonizing effect on *S. mutans* levels. Subjects in the probiotic group who did not colonize returned to pre-M18 levels of *S. mutans* after 3 days. Subjects who did colonize with *S. salivarius* M18 demonstrated a significant drop in *S. mutans* levels for up to 7 months of testing. Furthermore, of those subjects who did colonize with *S. salivarius* M18, 78% demonstrated *S. mutans* levels below $1 x10^3$ CFU/mL. During the trial, children were asked each month if they had experienced any ill effects and these self-reported adverse effects were recorded. Four cases of adverse reactions were reported, specifically, 3 events in the *S. salivarius* M18 group included a sore throat and 2 cases of chickenpox, while 1 bleeding gum event occurred in the placebo group. None of the adverse events were considered serious or related to the treatment. No subject left the trial as a result.

A randomized controlled clinical study was conducted in healthy male and female children (6 to 17 years) at risk for dental caries to assess the safety and tolerability of oral tablets containing *S. salivarius* M18 and to evaluate effects on dental caries formation (Di Pierro *et al.,* 2015). Subjects in the treatment group were orally supplemented once a day with slowly dissolving tablets containing *S. salivarius* M18 (>$1x10^9$ CFU/tablet) (n=38). The control group (n=38) did not receive any treatment for 90 days. Subjects were evaluated every 15 days by the study dentists for probiotic tolerability and dosing compliance; the occurrence of any side effects was reported by the subjects and/or their parents. Paraffin-stimulated whole saliva samples were collected from all subjects at baseline and on Day 90 to measure the levels of *S. mutans*, evaluate saliva pH and quantity, and detect the presence of dental plaque. Treatment with *S. salivarius* M18 for 90 days significantly reduced the chances of developing new cavities by 33% compared to placebo. Mutans streptococci and plaque control were also significantly reduced following *S. salivarius* M18 exposure when compared to baseline measurements. Tolerability was reported as “very good” and “good” in 35 out of 38 subjects, and “acceptable” in the remaining 3 subjects. No treatment-related side effects were reported in any of the subjects supplemented with *S. salivarius* M18, and the authors concluded that *S. salivarius* M18 demonstrated a “very good” safety profile.

A randomized controlled study was conducted in healthy male and female adults (20 to 60 years) with moderate and severe gingivitis and moderate periodontitis (Scariya *et al.,* 2015). Subjects were instructed to consume 2 lozenges containing *S. salivarius* M18 (>1x10^8 CFU/lozenge; n=7/sex) twice daily (*i.e.,* once in the morning and in the evening) for 30 days. Subjects in the control group (n=7/sex) did not consume any lozenges. Several clinical parameters were assessed at baseline, days 15, 30, 45, and 60, including supragingival plaque, gingival index, bleeding on probing, and probing pocket depth. In terms of safety parameters, none were specifically assessed in the study; using self-reporting, no adverse reactions were declared.

The above clinical studies demonstrate that *S. salivarius* M18 is well-tolerated and safely consumed by healthy children at up to 7.2x10^9 CFU per day for 90 days and by adults with moderate and severe gingivitis and moderate periodontitis at up to 2x10^8 CFU per day for 30 days.

6.4.2 Studies with *S. salivarius* K12

A comprehensive search of the scientific literature up to February 28, 2018 was conducted to identify studies investigating human exposure to *S. salivarius* K12. Eighteen studies conducted in adults, children, and infants using *S. salivarius* K12 were identified and in particular, 1 study specifically evaluated the safety of *S. salivarius* K12 (Burton *et al*., 2011). The remaining studies were not conducted to examine safety-related endpoints; however, the absence of reported adverse effects in these studies corroborate the safety of the intended use of *S. salivarius* M18 in food. Supplementation with up to 1.1x10^{10} CFU/day of *S. salivarius* K12 for up to 28 days was reported to be well-tolerated, and any reported adverse effects were either mild or were not related to *S. salivarius* K12 consumption. The results of these studies are summarized in Table 6.4.2-1.

Table 6.4.2-1 Summary of Human Studies with *Streptococcus salivarius* K12

Study Design	Population	Dose; Duration	Safety-Related Endpoints Measured	Safety-Related Results [a]	Reference
Safety Studies					
Randomized, double-blind, placebo-controlled, parallel arm	Adults (M&F), healthy	1.1 x 10^{10} CFU/day (n=27) or placebo (n=28); 28 days	• Vital signs, clinical chemistry, hematology, urinalysis • Self-reported questionnaires for oral and gastrointestinal health	• No changes in vital signs, clinical chemistry, or hematology parameters • Lower increase from baseline for urine specific gravity; however, end of treatment values for both groups were similar and within normal range • The number of AEs related to treatment was no different between groups (2 events in the probiotic group, 3 in the placebo) • No serious AE reported	Burton *et al*. (2011)
Other Studies					
Randomized, uncontrolled, parallel arm	Adults (M&F), healthy	7.0x10^4, 1.0x10^6, 2.0x10^7, 1.1x10^8, or 1.5x10^9 CFU/day (20/group); 14 days	• AE reporting	• No AE reported by subjects	Burton *et al.* (2010)
Single-arm	Adults (M&F), healthy	4x10^9 CFU/day (n=14); 3 days	• AE reporting	• No adverse symptoms reported by any subjects	Burton *et al.* (2006a)
Pilot study	Adult (M), healthy	4x10^{10} CFU/day (n=1); 3 days	• AE reporting	• No adverse symptoms reported	Horz *et al.* (2007)
Pilot, uncontrolled	Adults (M&F), rheumatoid arthritis (RA)	BLIS BioRestore™: 2x10^8 CFU/day *S. salivarius* K12, 8x10^8 CFU/day *L. acidophilus* L10, and 8x10^8 *B. lactis* B94 (n=12); 7 days	• Full blood count, renal and liver function tests	• No changes in full blood count or liver and renal function tests • 4 patients reported mild to moderate AEs; 1 flare of rheumatoid arthritis, 3 cases of GI disturbance	Lee *et al.* (2010)
Placebo-controlled, parallel arm	Adults (M&F), healthy with halitosis	>4x10^9 CFU/day (n=13) or placebo (n=10); 3 days	• None	• None	Burton *et al.* (2006b)

Table 6.4.2-1 Summary of Human Studies with *Streptococcus salivarius* K12

Study Design	Population	Dose; Duration	Safety-Related Endpoints Measured	Safety-Related Results [a]	Reference
Placebo-controlled	Adults (M&F), recurrent oral *Streptococcal* pharyngitis	5 x 10^9 CFU/day (n=20) or placebo (n=20); 90 days	• Side effects were recorded by physicians at biweekly appointments	• No side effects occurred in any of the subjects	Di Pierro *et al*. (2013)
Multi-center, open, controlled	Children (M&F), recurrent oral *streptococcal* disorders	1x10^9 CFU/day (n=31) or untreated control (n=30); 90 days	• Side effect reporting	• No side effects reported	Di Pierro *et al* (2014)
Observational, retrospective	Children (M&F), group A beta-hemolytic streptococci recurrent pharyngo-tonsillar infections	1 x 10^9 CFU/day (n=76) or untreated control (n=54); 90 days	• None	• None	Gregori *et al.* (2016)
Pilot, uncontrolled	Children (M&F), secretory otitis media	>1 x 10^9 CFU/day (n=22); 90 days	• Side effect reporting	• No treatment-related side effects reported	Di Pierro *et al.* (2015)
Multi-center, open, controlled	Children (M&F), recurrent pharyngeal streptococcal disease	>1 x 10^9 CFU/day (n=48) or untreated control (n=76); 90 days	• Side effect reporting	• No treatment-related side effects reported	Di Pierro *et al.* (2016a)
Multi-center, open, randomized, controlled	Children (M&F), healthy	>1 x 10^9 CFU/day (n=111) or untreated control (n=111); 6 months	• Side effect reporting	• No treatment-related side effects reported	Di Pierro *et al.* (2016b)
Retrospective	Children (M&F), non-recurrent streptococcal infection	>1 x 10^9 CFU/day (n=133); 6 months	• Side effect reporting	• No treatment-related side effects reported	Di Pierro *et al.* (2018)
Randomized, controlled	Children (M&F), high risk of acute rheumatic fever	2.5x10^9 CFU/school day (n=666) or placebo (n=648); 1 year	• None	• None	Doyle *et al.* (2017)
Multi-center, controlled	Children (M&F), recurrent oral *streptococcal* pharyngitis/ tonsillitis	5x10^9 CFU/day (n=45) or untreated control (n=20); 90 days	• Side effect and tolerability monitoring	• Very well-tolerated • No side effects reported	Di Pierro *et al.* (2012)
Single-arm	Children (gender not reported), scheduled to undergo ventilation tube placement surgery	1x10^{10} to 3.4x10^{10} CFU/ day; 10 days	• None	• None	Power *et al.* (2008)

Table 6.4.2-1 Summary of Human Studies with *Streptococcus salivarius* K12

Study Design	Population	Dose; Duration	Safety-Related Endpoints Measured	Safety-Related Results [a]	Reference
Randomized, controlled	Children (M&F), co-treated with chlorhexidine, halitosis	>1x10^9 CFU/ day + chlorhexidine (n=52) or chlorhexidine control (n=52); 90 days	• None	• None	Jamali *et al.* (2016)
Multi-center, randomized, double-blind, placebo-controlled	Infants (gender not reported), risk of acute otitis media	Formula containing a mixture of probiotics, providing 1x10^9 - 2x10^9 CFU/day *S. salivarius* K12; 12 months	• Anthropometric measurements • AE reporting	• Formula well-tolerated • No change in anthropometric measures of growth • 93.1% of AE not related to treatment (one incident of constipation considered related)	Cohen *et al*. (2013)

AE = adverse event; CFU = colony-forming units; F = female; GI = gastrointestinal, M = male.
[a] Unless stated otherwise, all reported effects are relative to control group(s).

6.5 Safety of Organism/Pathogenicity

6.5.1 Case Reports of Human Infection Related to *Streptococcus salivarius*

S. salivarius is a natural inhabitant of the oropharynx and the gastrointestinal tract, and in rare instances it can be an opportunistic pathogen in individuals with a serious underlying condition. Numerous case reports of infection related to *S. salivarius* have been reported in the literature and comprehensive reviews of these studies and the pathogenic potential of the species *S. salivarius* have been previously reported in Section IV.E.1 of GRN 591 (U.S. FDA, 2016). Case reports identified in the literature are almost exclusively iatrogenic in nature with incidences typically related to infection following surgical intervention with poor hygiene control, major tissue trauma, or occurred in immunocompromised individuals. The Expert Panel evaluation of data and information presented in GRN 591 (U.S. FDA, 2016) also discussed findings from Dr. Jonathan Carapetis of the University of Western Australia, a qualified expert in Streptococcal disease, pediatric infectious diseases, and public health. Dr. Carapetis concluded the following:

> "*From the information provided to me there is no reason to believe that the risk of human infection associated with administration of S. salivarius will be any greater than that of other probiotic organisms and it appears from an examination of patient outcomes that any infection that results may be less clinically significant than other commonly used probiotic organisms*".

GRN 591 (U.S. FDA, 2016) also discusses additional observations by the Expert Panel, who noted:

> "*S. salivarius is a dominant species within the oral microflora and is present in all individuals from birth and throughout life. In humans (and likely most mammals), direct exposure of S. salivarius to the systemic circulation through minor and major trauma to the oral mucosa therefore occurs on a routine basis in all individuals, across all age groups and population types, including immunocompromised persons. Ubiquitous transfer of S. salivarius isolates between individuals through normal social interactions is without adverse effects* […]".

An updated literature search up to 01 March 2018 using the search terms "*Streptococcus salivarius*" and "pathogen*", "infect*", "virulen*", "toxicogen*", "bacteremi*", "septicemi*", or "meningitis" identified 1 new study and 2 new reviews characterizing *S. salivarius* isolates in clinical infections (Dowling *et al.,* 2015; Abbouda *et al.,* 2016; Zorrilla-Vaca *et al.,* 2018). Findings from these publications continue to support conclusions that the pathogenicity of *S. salivarius* is opportunistic in nature and does not appear to differ from that of other innocuous microbial species (*e.g.*, *Lactobacillus*, bifidobacteria) that are used in food.

6.5.2 Bioinformatic Analyses for Virulence Determinants and Antibiotic Resistance Factors

A bioinformatic search of the annotated assembly of the *S. salivarius* M18 whole genome (AGBV00000000) was conducted using the online tool PATRIC v. 3.5.7 (https://www.patricbrc.org/). The genome assembly, created using Newbler v. 2.3, was evaluated for virulence genes using PATRIC online software, which conducts BLAST searches of the genome against lists of putative virulence genes maintained by PATRIC and originating from 3 manually curated online databases: PATRIC VF (https://www.patricbrc.org/), VFDB (http://www.mgc.ac.cn/VFs/main.htm), and Victors (http://www.phidias.us/victors/). PATRIC subsystem analyses of the genome also included identification of antibiotic resistance factors against genes maintained in the ARDB and CARD database. No genes encoding known streptococcal toxins (*e.g.*, streptolysin O and S) or antibiotic resistance determinants were identified. A total of 50 genes with homology to putative virulence genes were identified in the genome and megaplasmid of M18 (see Table D1 in Appendix C). Most of these genes were defensive or non-classical virulence factors, such as determinants related to

transcription, translation, post-translational modifications, ribosomal structure and biogenesis, replication, recombination and repair, cell motility, signal transduction mechanisms, intra- and extracellular transportation, metabolism and transport of lipids, coenzymes, amino acids and carbohydrates, signal transduction mechanisms, cell cycle control, cell division and chromosome partitioning, protein turnover and chaperones, energy production and conversion and membrane biogenesis. For interpretation of the virulence hits, comparisons were conducted against the whole genome and megaplasmid of the GRAS strain *S. salivarius* K12. Of the 50 'virulence genes' identified for *S. salivarius* M18 (see Appendix C), only 4 genes were unique to M18 (Table 6.6.2-1) (*i.e.*, not present within the genome of *S. salivarius* K12). BLAST searches of the FASTA protein sequences against non-redundant sequences in the Genbank database identified all the genes as being commonly expressed by variety of non-pathogenic *Streptococcus* sp.

Table 6.5.2-1 PATRIC *vs.* 3.5.7 Subcategory Analyses of the Annotated Genomes of *Streptococcus salivarius* M18 and *Streptococcus salivarius* K12 - Specialty Genes Categorized as Virulence Factors

***S. salivarius* M18**		***S. salivarius* K12**	
Source	**No. of Hits to Homologous Genes**	**Source**	**No. of Hits to Homologous Genes**
VFDB	2 (whole genome)	VFDB	2 (whole genome)
Victors	47 (whole genome) 1 (plasmid)	Victors	49 (whole genome) 1 (plasmid)
Genes Specific to *S. salivarius* M18			
Source ID	**Source Organism**	**Product**	
Victors - 15900188	*Streptococcus pneumoniae* TIGR4	Glycerol dehydrogenase (EC 1.1.1.6)	
Victors - 15900208	*Streptococcus pneumoniae* TIGR4	DNA polymerase III polC-type (EC 2.7.7.7)	
Victors - 15900547	*Streptococcus pneumoniae* TIGR4	C5a peptidase precursor (EC 3.4.21.-)	
Victors - 116516119	*Streptococcus pneumoniae* D39	Pyruvate oxidase (EC 1.2.3.3)	

Additional bioinformatics analyses of the *S. salivarius* M18 gene assembly for pathogenicity (PathogenFinder 1.1) and antibiotic resistance (ResFinder 3.0) were conducted using online *in silico* tools hosted by the Center for Genomic Epidemiology (CGE). PathogenFinder utilizes a validated method for predicting the pathogenicity of novel bacteria by comparing protein families from the strain of investigation to a protein family database (PFDB) composed of groups of proteins or protein families associated with pathogenic or non-pathogenic organisms (Cosentino *et al*., 2013). The reference database was constructed using 372 bacterial genomes tagged as pathogenic and 513 tagged as non-pathogenic. The prediction method is unique in that the model was developed without prior analysis of the proteins in the training set, but by tagging the training organisms as pathogenic or non-pathogenic and identifying protein families that were frequently found in pathogenic or non-pathogenic bacteria. As reported by Cosentino *et al*. (2013), PathogenFinder performs better than other pathogenicity prediction models reported in the literature which rely on taxonomy and sequence similarity to small sets of genes known to be associated with bacterial pathogenesis. Analyses of the *S. salivarius* M18 assembly using PathogenFinder 1.1. identified the strain as non-pathogenic (Figure 6.6.2-2). Two hits to protein families characteristic of *S. gordonii* and *S. suis* were identified as an integral membrane protein with unknown function (ABV09764) and a ribosomal protein (ABP89047). Both proteins are common to the species *salivarius* and are not expected to confer undesirable phenotypes.

Table 6.5.2-2 Bioinformatic Analyses of *Streptococcus salivarius* M18 Whole Genome Using PathogenFinder 1.1

Project ID	Accession ID	Organisms	Protein Function	Protein ID	% Identity
13773	CP000419	*S. thermophilus* LMD-9	Na+/xyloside symporter or related transporter	ABJ66540	97.32
13163	CP000024	*S. thermophilus* CNRZ1066	cobalamin biosynthesis protein	AAV61900	97.85
13162	CP000023	*S. thermophilus* LMG 18311	replication initiator protein	AAV61548	97.85
13162	CP000023	*S. thermophilus* LMG 18311	urease accessory protein	AAV60009	96.42
13162	CP000023	*S. thermophilus* LMG 18311	ABC transporter membrane-spanning protein	AAV60011	96.14
13162	CP000023	*S. thermophilus* LMG 18311	urease accessory protein	AAV60007	97.05
13162	CP000023	*S. thermophilus* LMG 18311	cell division ATP-binding protein	AAV60783	99.13
13162	CP000023	*S. thermophilus* LMG 18311	type I restriction-modification system specificity subunit	AAV60651	97.09
66	CP000725	*S. gordonii* str. Challis substr. CH1	membrane protein, putative	ABV09764	99.51
13162	CP000023	*S. thermophilus* LMG 18311	peptidyl-tRNA hydrolase	AAV59735	98.41
13162	CP000023	*S. thermophilus* LMG 18311	unknown protein	AAV60389	96.86
13773	CP000419	*S. thermophilus* LMD-9	Predicted membrane protein	ABJ66893	96.41
13162	CP000023	*S. thermophilus* LMG 18311	urease accessory protein	AAV60006	99.33
13773	CP000419	*S. thermophilus* LMD-9	Ribonuclease III family protein	ABJ65467	96.24
17153	CP000407	*S. suis* 05ZYH33	SSU ribosomal protein S19P	ABP89047	100.0
13162	CP000023	*S. thermophilus* LMG 18311	conserved hypothetical protein	AAV61555	99.01

The input organism was predicted as non-human pathogen

Probability of being a human pathogen	0.279
Input proteome coverage (%)	0.76
Matched Pathogenic Families	2
Matched Not Pathogenic Families	14

Identification of antibiotic resistance genes was conducted by *in silico* analyses of the whole genome sequence of *S. salivarius* M18 using ResFinder 3.0. ResFinder is a web-based method developed using 1862 Genbank test files containing 1411 different resistance genes from 12 different antimicrobial classes. The database is the largest manually curated database of antibiotic resistance genes and is updated on a continual basis as new antibiotic resistance genes are identified. The current version covers horizontally acquired resistance genes, and validation of the method was established using antimicrobial susceptibility data for 23 isolates covering 5 different species (*E. coli, K. pneumonia, S. enterica, S. aureus, V. cholerae*) (Zankari *et al*., 2012). Almost complete agreement between *in silico* predictions and the phenotypic testing was found. The whole genome sequence of *S. salivarius* M18 was evaluated by ResFinder 3.0. For conservative purposes the default identity (90%) and coverage (60%) settings were changed to 60% and 40%, respectively. No positive alignments to potential antimicrobial resistance genes were identified. Antimicrobial resistance also was tested on the CGE website using ResFinderFG 1.0, which identifies resistance phenotype based on a functional metagenomic antibiotic resistance determinants database. Genes hosted in the database are currently based on results of functional metagenomic studies that were analyzed in human fecal samples, sewage, latrines, and soils (Sommer *et al*., 2009; Fu *et al*., 2012; Moore *et al*., 2013; Forsberg *et al.*, 2014; Seemann, 2014; Pehrsson *et al*., 2016). Analyses of the *S. salivarius* M18 whole genome sequence using ResFinderFG 1.0 resulted in 1 match to an uncultured clone (Genbank KF629717.1) with putative resistance to trimethoprim. A BLAST search of the cloned fragment identified hits with >90% identity to a majority of the *S. salivarius* genomes that have been sequenced

indicating that the genotype is unlikely to represent an acquired resistance trait. For example, as reported by Applebaum (2002), resistance to trimethoprim in *Streptococcus pneumoniae* attributed to mutations in the dihydrofolate reductase gene resulted in reduced affinity of trimethoprim to its target enzyme, dihydrofolate reductase.

Figure 6.5.2-1 Outputs from *In Silico* Analyses of *Streptococcus salivarius* M18 Whole Genome Sequence Using (A) ResFinder 3.0 and (B) ResFinderFG 1.0

A)

B)

functional genomics						
Hit name	Identity	Query/HSP	Contig	Position in contig	Drug treatment	Accession no.
dfr	98.72	546/546	AGBV01000004.1	842750..843295	Trimethoprim	KF629717.1

6.5.3 Discussion and Conclusion

S. salivarius is a species that is indigenous to the oropharynx, and colonization of the oropharynx of humans has been reported to occur by 1 month of age (Hegde and Munshi, 1998). In adults, the species is a dominant microorganism in the oral cavity representing up to a third of the total *Streptococcus* counts (Frandsen *et al.*, 1991). In addition to the fact that exposure to the strain occurs in all humans across all ages, including healthy and unhealthy individuals, transfer of 'foreign' strains between individuals is expected through normal social interactions on a continual basis (Kort *et al*., 2014). A comprehensive review of the literature demonstrated that strains of *S. salivarius* have been associated with rare cases of opportunistic infections. Iatrogenic cases of meningitis from improper surgical practice represented one of the most common causes of opportunistic infections reported in the literature. This route of exposure is not relevant to food applications and it also is noteworthy that no cases of spontaneous meningitis have been reported in otherwise healthy individuals. Clinical cases of opportunistic infection have been successfully treated with antibiotics, and few instances of significant sequelae have been reported to result from *S. salivarius* infection (Wilson *et al.*, 2012). Case reports of opportunistic infection with *S. salivarius* are comparable to similar reports of opportunistic infections involving lactobacilli or bifidobacteria, including strains commonly used in fermented foodstuffs. Borriello *et al.* (2003) conducted a review of infections involving *Lactobacillus* species in Finland and identified a background level of 10 to 20 cases per year between the years 1995 and 1999. The number of infections reported during this period occurred against a notable increase in the consumption of probiotics. The findings of this study suggest that an increase in the use of probiotic products has not led to an increase in opportunistic infections.

S. salivarius does not contain any major virulence factors that have been described for pathogenic streptococci (see Section 6.5.2), and extensive genomic and bioinformatic analyses of various clinical isolates have not identified genetically controlled pathogenicity traits. For example, Delorme *et al.* (2007) evaluated the relationship between the commensal strains of *S. salivarius* and the strains of *S. salivarius* associated with invasive infections using sequence analyses and multi-locus sequence typing. The authors reported that the infection-associated strains could not be distinguished from the commensal strains, thus suggesting that the infection-associated strains were opportunistic, rather than pathogenic in nature. In a subsequent study by the authors, gene sequencing and annotation was used to characterize *S. salivarius* CCHSS3, a clinical isolate obtained from a human blood sample (Delorme *et al*., 2011). The authors reported that "*no known virulence factor, antibiotic resistance determinant, or putative genomic island representative of the accessory genomes of pathogenic species was found*" (Delorme *et al.*, 2011). Finally, the results of toxicological and human safety studies, including long-term administration to sensitive population groups such as infants (Cohen *et al.,* 2013) have not resulted in adverse effects or changes in biochemical/hematological indices suggestive of infection.

Based on the above review it was concluded that there is no evidence to suggest that introduction of *S. salivarius* strains to the food supply, at use-levels consistent with those currently used for other probiotic food preparations, would be unsafe.

6.6 Antibiotic Resistance

The antibiotic resistance of *S. salivarius* M18 was evaluated by independent laboratories using the antibiotic disc sensitivity method. Two independent studies were conducted in accordance with Clinical and Laboratory Standards Institute (CLSI) protocols. As shown in Table 6.6-1, *S. salivarius* M18 was sensitive to penicillin, ampicillin, amoxicillin, vancomycin, erythromycin, tetracycline, ofloxacin, clindamycin, ceftriaxone, and chloramphenicol. There were no CLSI guidelines for breakpoints for kanamycin, gentamycin, or streptomycin.

Table 6.6-1 Antibiotic Resistance Testing of *Streptococcus salivarius* M18

Antibiotic	Minimum Inhibitory Concentration (mg/L)	
	E-test[a]	Agar Dilution[b]
Ampicillin	0.06 (S)[c]	0.03 (S)[c]
Amoxicillin	NT	0.015 (S)
Ceftriaxone	NT	0.5 (S)
Chloramphenicol	NT	2 (S)
Clindamycin	0.03 (S)	0.06 (S)
Erythromycin	0.06 (S)	0.25 (S)
Gentamycin	16 (NIS)	32 (NIS)
Kanamycin	NT	64 (NIS)
Ofloxacin	NT	2 (S)
Penicillin	NT	0.06 (S)
Streptomycin	NT	32 (NIS)
Tetracycline	0.25 (S)	0.25 (S)
Vancomycin	0.5 (S)	0.5 (S)

(NIS) = no interpretive standard; NT = not tested; (S) = susceptible.
[a] The E-test method was performed by Environmental Science and Research (ESR) - Communicable Disease Group.
[b] The agar dilution method performed by Southern Community Laboratories, Dunedin, New Zealand, according to the European Committee for Antimicrobial Susceptibility Testing (2003).
[c] Minimum inhibitory concentration (MIC) interpretation was determined in accordance to Clinical and Laboratory Standards Institute guidelines (M100-S19, Jan 2009).

6.7 Additional Metabolic Considerations

6.7.1 Production of Antimicrobials

Streptococci are a predominant species in the oral cavity (Tagg, 2004), and the production of bacteriocins by naturally occurring oral streptococci is widespread (Burton *et al.,* 2013a). The production of bacteriocins is ubiquitous among gram positive bacteria, and almost all bacteriocins have a net positive charge at neutral or slightly acidic pH, and often contain regions that are hydrophobic and/or amphiphilic; physicochemical properties that are critical for their bactericidal activities (Eijsink *et al.*, 2002). One class of bacteriocins, the lantibiotics, are produced by closely-related gram-positive bacteria (Upton *et al.*, 2001) and are among the most well-studied group of bacteriocins. Subtype A lantibiotics are linear, amphipathic, small cationic peptides, of which one of the most well-known compounds is nisin (subtype AI); a compound that has a long history of use in the food industry. In the U.S., nisin is permitted for use on casings for frankfurters and on cooked meat and poultry products as an antimicrobial agent (GRN 65) (U.S. FDA, 2001). Salivaricin lantibiotics (subtype AII) are also well-characterized compounds and are widely produced by the *S. salivarius* species. Dierksen *et al.* (2007), for example, detected the gene encoding salivaricin A2 (*salA*) in 11 out of 18 *S. salivarius* strains tested by PCR analysis. Similarly, production of the streptococcal lantibiotics salivaricin A, B, streptin, and SA-FF22 were detected in 9 of 28 *S. salivarius* strains tested (Wescombe *et al.*, 2006).

S. salivarius M18 produces 4 bacteriocins, the subtype AII lantibiotics salivaricins A2, 9, MPS, and M (Heng *et al.,* 2011), whereas *S. salivarius* K12 only produces 2 bacteriocins, salivaricin A2 and salivaricin B (Wescombe *et al.*, 2006). The well-characterized bacteriocins salivaricin A2 and 9 are active against *S. pyogenes* and other pathogens that infect the upper respiratory tract (Wescombe *et al.,* 2011), and salivaricin MPS, while less characterized, has also demonstrated activity against *S. pyogenes* (Dodd, 1999). Salivaricin M, the newest of the *S. salivarius* M18 bacteriocins is active against mutans streptococci (Heng *et al.,* 2011).

Like most bacteriocins, subtype A lantibiotics (*e.g.,* nisin (food additive), salivaricin) operate *via* surfactant effects, disrupting cell membranes *via* pore formation, which in turn leads to dissipation of proton motive forces, adenosine triphosphate (ATP) depletion, and leakage of intracellular contents (Cleveland *et al.*, 2001). Although the specific mechanism of action by which these molecules induce pore formation is complex and unique to each bacteriocin, in general, pore formation occurs through electrostatic interactions between positively charged peptide sequences and anionic lipids that are abundantly present in on the cell membrane (Eijsink *et al.*, 2002). This mechanism of action is distinct from those of pharmacological antibiotics, which act through highly specific drug-protein interactions that target specific and critically conserved protein structures (*e.g.*, ribosomal enzymes). Due to the high specificity of these drug-protein interactions, antibiotics induce antibacterial effects at low concentrations, typically against a fairly wide-spectrum of organisms. Thus, gene mutation events in the gene encoding the antibiotic target protein, which frequently occur in bacteria, can lead to antibiotic resistance, and the transfer of this resistance to other organisms. Given the differences, however, in the mechanisms of action between antibiotics and lantibiotic bacteriocins, the presence of lantibiotics in the food supply would not result in the development or propagation of antibiotic resistance against clinically-important antibiotics.

6.8 Pariza Decision Tree – Determining the Safety of Microbial Cultures for Consumption

An evaluation of the safety of *S. salivarius* M18 for human consumption was conducted using the Decision Tree approach described by Pariza and colleagues (Pariza *et al*., 2015).

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding.)

YES. The whole genome and plasmid has been sequenced and is deposited in a publicly available database. *S. salivarius* M18 also has been subject to classical phenotypic and genotypic characterization.

2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)

YES.

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)

YES. Bioinformatic analyses of the whole genome have demonstrated that the strain is free of classical *Streptococcus* virulence factors and is absent of genes potentially conferring pathogenic traits to the organism.

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)

YES. The absence of functional and transferable antibiotic resistance genes has been demonstrated using bioinformatic analyses and classical *in vitro* analyses using the E-test and agar dilution assays.

5. Does the strain produce antimicrobial substances? (If NO, go to 6. If YES, go to 15.)

NO. The strain is known to produce bacteriocins (*e.g.*, salivaricins) that are antagonistic to *Streptococcus* sp.; however, there is no documented evidence of the species *S. salivarius* producing antibiotics with structure activity behaviors that would be of clinical importance.

6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a or 7b. If NO, go to 8a.)

NO.

8a. For strains to be used in human food was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? (If YES, go to 9a. If NO, go to 13a.)

NO; however, the strain is a human commensal obtained from saliva sample of healthy volunteer therefore it is considered appropriate to proceed to 9a.

9a. For strains to be used in human food: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts? (If YES, go to 10a. If NO, go to 13a.)

YES. *S. salivarius* K12 has GRAS status as described in GRN 591 (U.S. FDA, 2016).

10a. For strains to be used in human food: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food? (If YES, go to 11a. If NO, go to 13a.)

YES.

11a. For strains to be used in human food: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12a. If YES, go to 13a.)

NO. Ingestion of *S. salivarius* strains is ubiquitous in the general population, including at risk individuals, through consumption of saliva (*i.e., S. salivarius* is typically present at levels of around 1×10^7 CFU/mL of saliva). Transfer of *S. salivarius* strains between humans through normal social interactions also is not associated with safety concerns.

12a. For strains to be used in human food: Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14a. If YES, go to 13a.)

NO. Food-uses are consistent with those described under GRN 591 (U.S. FDA, 2016) for *S. salivarius* K12 and the expanded intake of the species is not expected.

13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? If YES, go to 15. If NO, go to 14a.)

NO. There are no anticipated physiological effects of consuming the strain outside of potential transient changes in the oral microflora composition. These strain specific effects of *S. salivarius* M18 have been evaluated in human colonization studies and no adverse changes in the oral flora composition or other adverse physiological effects have been reported in the studies.

14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

6.9 Expert Panel Evaluation

BLIS Technologies Ltd. has concluded that *S. salivarius* M18 as described herein meeting appropriate food grade specifications and manufactured consistent with current Good Manufacturing Practices, is GRAS for use as an ingredient in specified conventional food and beverage products, as described in Part 1.3, on the basis of scientific procedures.

This GRAS determination is based on data generally available in the public domain pertaining to the safety of *S. salivarius* M18 and on a unanimous opinion among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of Dr. Joseph Borzelleca, Ph.D., (Virginia Commonwealth University School of Medicine), Dr. Robert Nicolosi, Ph.D., (University of Massachusetts Lowell), and Dr. Michael Pariza, Ph.D. (University of Wisconsin). A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of *S. salivarius* M18 is presented in Appendix D.

6.10 Conclusions

In conclusion, data and information presented herein demonstrates that the intended uses of *S. salivarius* M18, meeting appropriate food-grade specifications and manufactured according to cGMP are safe. The data and information summarized in this report also demonstrate that the intended uses of *S. salivarius* M18 described herein would be GRAS based on scientific procedures.

Part 7. §170.255 List of Supporting Data and Information

Abbouda A, Abicca I, Alió JL (2016). Infectious keratitis following corneal crosslinking: A systematic review of reported cases: management, visual outcome, and treatment proposed. Semin Ophthalmol 31(5):485-491. DOI:10.3109/08820538.2014.962176.

Abdelgadir WS, Hamad SH, Møller PL, Jakobsen M (2001). Characterisation of the dominant microbiota of Sudanese fermented milk *Rob*. Int Dairy J 11(1&2):63-70.

Appelbaum PC (2002). Resistance among *Streptococcus pneumoniae*: implications for drug selection. Clin Infect Dis 34(12):1613-1620.

Arumugam M, Raes J, Pelletier E, Le Pasilier D, Yamada T, Mende DR, *et al*. (2011). Enterotypes of the human gut microbiome. Nature 473(7346):174-180.

Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J, *et al*. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci U S A 107(44):18933-18838.

Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM (2007). Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2(3):285-295.

Booijink CC, El-Aidy S, Rajilić-Stojanović M, Heilig HG, Troost FJ, Smidt H, *et al*. (2010). High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 12(12):3213-3227. DOI:10.1111/j.1462-2920.2010.02294.x.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara, M *et al*. (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 36(6):775-780.

Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, *et al*. (2012). Food fermentations: microorganisms with technological beneficial use. Int J Food Microbiol 154(3):87-97 [Erratum, 156(3):301] .DOI: 10.1016/j.ijfoodmicro.2011.12.030.

Burton JP, Moore CJ, Chilcott CN, Tagg JR (2006a). Safety assessment of the oral cavity probiotic *Streptococcus salivarius* K12. Appl Environ Microbiol 72(4):3050-3053.

Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR (2006b). A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J Appl Microbiol 100(4):754-764.

Burton JP, Chilcott CN, Wescombe PA, Tagg JR (2010). Extended safety data for the oral cavity probiotic *Streptococcus salivarius* K12. Probiotics Antimicrob Proteins 2(3):135-144.

Burton JP, Cowley S, Simon RR, McKinney J, Wescombe PA, Tagg JR (2011). Evaluation of safety and human tolerance of the oral probiotic Streptococcus salivarius K12: a randomized, placebo-controlled, double-blind study. Food Chem Toxicol 49(9):2356-2364.

Burton JP, Wescombe PA, Macklaim JM, Chai MH, MacDonald K, Hale JD, *et al*. (2013a). Persistence of the oral probiotic *Streptococcus salivarius* M18 is dose dependent and megaplasmid transfer can augment their bacteriocin production and adhesion characteristics. PLoS One 8(6):e65991 [10pp]. DOI:10.1371/journal.pone.0065991.

Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JD, *et al*. (2013b). Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. J Med Microbiol 62(6):875-884. DOI:10.1099/jmm.0.056663-0.

Bylund G, editor (1995). Chapter 10: Cultures and starter manufacture. In: *Dairy Processing Handbook*. Lund, Sweden: Tetra Pak Processing Systems AB, pp. 233-240.

Callon C, Millet L, Montel MC (2004). Diversity of lactic acid bacteria isolated from AOC Salers cheese. J Dairy Res 71(2):231-244.

CDC (2006). *Analytical and Reporting Guidelines: The National Health and Nutrition Examination Survey (NHANES)*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/nhanes_analytic_guidelines_dec_2005.pdf.

CDC (2009). *National Health and Nutrition Examination Survey (NHANES): 2005-2006*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/nhanes/nhanes2005-2006/nhanes05_06.htm.

Chen YY, Weaver CA, Burne RA (2000). Dual functions of Streptococcus salivarius urease. J Bacteriol 182(16):4667-4669. Cited In: Lee *et al*., 2009 [Ref. #6].

Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001). Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71(1):1-20.

Cohen R, Martin E, de La Rocque F, Thollot F, Pecquet S, Werner A, *et al*. (2013). Probiotics and prebiotics in preventing episodes of acute otitis media in high-risk children: a randomized, double-blind, placebo-controlled study. Pediatr Infect Dis J 32(8):810-814. DOI:10.1097/INF.0b013e31828df4f3.

Cosentino S, Voldby Larsen M, Møller Aarestrup F, Lund O (2013). PathogenFinder--distinguishing friend from foe using bacterial whole genome sequence data. PLoS One 8(10):e77302 [11pp]. DOI:10.1371/journal.pone.0077302. [Erratum, 8(12)]. DOI:10.1371/annotation/b84e1af7-c127-45c3-be22-76abd977600f.

Dalidowitz C (2005). Fortified breast milk safety. J Am Diet Assoc 105(10):1572-1573.

Delisle AL (1976). Activity of two Streptococcus mutans bacteriocins in the presence of saliva, levan, and dextran. Infect Immun 13(2):619-626.

Delorme C, Poyart C, Ehrlich SD, Renault P (2007). Extent of horizontal gene transfer in evolution of Streptococci of the salivarius group. J Bacteriol 189(4):1330-1341.

Delorme C, Guedon E, Pons N, Cruaud C, Couloux A, Loux V, *et al*. (2011). Complete genome sequence of the clinical *Streptococcus salivarius* strain CCHSS3. J Bacteriol 193(18):5041-5042.

Delorme C, Abraham AL, Renault P, Guédon E (2015). Genomics of Streptococcus salivarius, a major human commensal. Infect Genet Evol 33:381-392. DOI:10.1016/j.meegid.2014.10.001.

Di Pierro F, Donato G, Fomia F, Adami T, Careddu D, Cassandro C, *et al*. (2012). Preliminary pediatric clinical evaluation of the oral probiotic *Streptococcus salivarius* K12 in preventing recurrent pharyngitis and/or tonsillitis caused by *Streptococcus pyogenes* and recurrent acute otitis media. Int J Gen Med 5:991-997. DOI:10.2147/IJGM.S38859.

Di Pierro F, Adami T, Rapacioli G, Giardini N, Streitberger C (2013). Clinical evaluation of the oral probiotic *Streptococcus salivarius* K12 in the prevention of recurrent pharyngitis and/or tonsillitis caused by *Streptococcus pyogenes* in adults. Expert Opin Biol Ther 13(3):339-343. DOI:10.1517/14712598.2013.758711.

Di Pierro F, Colombo M, Zanvit A, Risso P, Rottoli AS (2014). Use of *Streptococcus salivarius* K12 in the prevention of streptococcal and viral pharyngotonsillitis in children. Drug Healthc Patient Saf 6:15-20.

Di Pierro F, Zanvit A, Nobili P, Risso P, Fornaini C (2015). Cariogram outcome after 90 days of oral treatment with *Streptococcus salivarius* M18 in children at high risk for dental caries: results of a randomized, controlled study. Clin Cosmet Investig Dent 7:107-113. DOI:10.2147/CCIDE.S93066.

Di Pierro F, Colombo M, Zanvit A, Rottoli AS (2016a). Positive clinical outcomes derived from using *Streptococcus salivarius* K12 to prevent streptococcal pharyngotonsillitis in children: a pilot investigation. Drug Healthc Patient Saf 8:77-81.

Di Pierro F, Colombo M, Giuliani MG, Danza ML, Basile I, Bollani T, *et al*. (2016b). Effect of administration of *Streptococcus salivarius* K12 on the occurrence of streptococcal pharyngo-tonsillitis, scarlet fever and acute otitis media in 3 years old children. Eur Rev Med Pharmacol Sci 20(21):4601-4606.

Di Pierro F, Risso P, Poggi E, Timitilli A, Bolloli S, Bruno M, *et al*. (2018). Use of Streptococcus salivarius K12 to reduce the incidence pharyngo-tonsillitis and acute otitis media in children: a retrospective analysis in not-recurrent pediatric subjects. Minerva Pediatr (Jan. 11). DOI:10.23736/S0026-4946.18.05182-4.

Dierksen KP, Moore CJ, Inglis M, Wescombe PA, Tagg JR (2007). The effect of ingestion of milk supplemented with salivaricin A-producing *Streptococcus salivarius* on the bacteriocin-like inhibitory activity of streptococcal populations on the tongue. FEMS Microbiol Ecol 59(3):584-591.

Dodd SJ (1999). *A Saliva Model to Demonstrate Streptococcus salivarius BLIS Production and Characterisation of Salivaricin MPS [M.S. Thesis]*. Dunedin, New Zealand: University of Otago. Cited In: Heng *et al*., 2011.

Dowling C, Lim C, Kerr N, Rao S, Robson D, Macdonald P, *et al*. (2015). Dental-associated viridans streptococci bloodstream infection in patients with continuous-flow ventricular assist devices. Heart Lung Circ 24(Suppl. 3): S202 [Abstract no 214].

Doyle H, Pierse N, Tiatia R, Williamson D, Baker M, Crane J (2017). The effect of the oral probiotic Streptococcus salivarius (K12) on group A Streptococcus pharyngitis: a pragmatic trial in schools. Pediatr Infect Dis J (Nov. 15): [Epub ahead of print]. DOI: 10.1097/INF.0000000000001847.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. (question no EFSA-Q- 2005-293, adopted on 19 November 2007 by European Food Safety Authority). EFSA J 587:1-16. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/587.htm.

Eijsink VG, Axelsson L, Diep DB, Håvarstein LS, Holo H, Nes IF (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Van Leeuwenhoek 81(1-4):639-654.

FAO/WHO (2002). *Guidelines for the Evaluation of Probiotics in Food*. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation in Food, Apr. 30-May, 1, 2002, London, Ont. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO) / World Health Organization (WHO). Available at: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Farrow JA, Collins MD (1984). DNA base composition, DNA-DNA homology and long-chain fatty acid studies on streptococcus thermophilus and *Streptococcus salivarius*. J Gen Microbiol 130(2):357-362.

Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002). Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68(1):219-226.

Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N, *et al*. (2014). Bacterial phylogeny structures soil resistomes across habitats. Nature 509(7502):612-616. DOI: 10.1038/nature13377.

Frandsen EV, Pedrazzoli V, Kilian M (1991). Ecology of viridans streptococci in the oral cavity and pharynx. Oral Microbiol Immunol 6(3):129-133.

Fu L, Niu B, Zhu Z, Wu S, Li W (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28(23):3150-3152. DOI:10.1093/bioinformatics/bts565.

Gibbons RJ, van Houte J (1975). Bacterial adherence in oral microbial ecology. Annu Rev Microbiol 29:19-44.

Gregori G, Righi O, Risso P, Boiardi G, Demuru G, Ferzetti A, *et al*. (2016). Reduction of group A beta-hemolytic streptococcus pharyngo-tonsillar infections associated with use of the oral probiotic *Streptococcus salivarius* K12: a retrospective observational study. Ther Clin Risk Manag 12:87-92. DOI:10.2147/TCRM.S96134.

Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, Stuknyte M, Karp M, *et al*. (2010). Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl Environ Microbiol 76(12):3948-3958.

Hakalehto E, Vilpponen-Salmela T, Kinnunen K, von Wright A (2011). Lactic acid bacteria enriched from human gastric biopsies. ISRN Gastroenterol 2011:Article ID 109183 [4pp]. DOI:10.5402/2011/109183.

Hamada S, Ooshima T, Torii M, Imanishi H, Masuda N, Sobue S, *et al*. (1978). Dental caries induction in experimental animals by clinical strains of *Streptococcus mutans* isolated from Japanese children. Microbiol Immunol 22(6):301-314.

Hasman H, Saputra D, Sicheritz-Ponten T, Lund O, Svendsen CA, Frimodt-Møller N, *et al*. (2014). Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. J Clin Microbiol 52(1):139-146 [Erratum, 52(8):3136]. DOI:10.1128/JCM.02452-13.

Hayacibara MF, Koo H, Vacca-Smith AM, Kopec LK, Scott-Anne K, *et al*. (2004). The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. Carbohydr Res 339(12):2127-2137.

Hegde S, Munshi AK (1998). Influence of the maternal vaginal microbiota on the oral microbiota of the newborn. J Clin Pediatr Dent 22(4):317-321.

Heikkilä MP, Saris PE (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. J Appl Microbiol 95(3):471-478.

Heng NC, Haji-Ishak NS, Kalyan A, Wong AY, Lovric M, Bridson JM, *et al*. (2011). Genome sequence of the bacteriocin-producing oral probiotic *Streptococcus salivarius* strain M18. J Bacteriol 193(22):6402-6403. DOI:10.1128/JB.06001-11.

Horz HP, Meinelt A, Houben B, Conrads G (2007). Distribution and persistence of probiotic *Streptococcus salivarius* K12 in the human oral cavity as determined by real-time quantitative polymerase chain reaction. Oral Microbiol Immunol 22(2):126-130.

ILSI (1995). A scientific basis for regulations on pathogenic microorganisms in foods. Dairy Food Environ Sanit 15(5):301-308.

Ishijima SA, Hayama K, Burton JP, Reid G, Okada M, Matsushita Y, *et al*. (2012). Effect of *Streptococcus salivarius* K12 on the *in vitro* growth of *Candida albicans* and its protective effect in an oral candidiasis model. Appl Environ Microbiol 78(7):2190-2199. DOI:10.1128/AEM.07055-11.

Jamali Z, Aminabadi NA, Samiei M, Sighari Deljavan A, Shokravi M, Shirazi S (2016). Impact of chlorhexidine pretreatment followed by probiotic *Streptococcus salivarius* strain K12 on halitosis in children: a randomised controlled clinical trial. Oral Health Prev Dent 14(4):305-313. DOI:10.3290/j.ohpd.a36521.

Kirjavainen PV, Apostolou E, Arvola T, Salminen SJ, Gibson GR, Isolauri E (2001). Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. FEMS Immunol Med Microbiol 32(1):1-7.

Kort R, Caspers M, van de Graaf A, van Egmond W, Keijser B, Roeselers G (2014). Shaping the oral microbiota through intimate kissing. Microbiome 17(2):41 [8pp]. DOI:10.1186/2049-2618-2-41.

Larsen MV, Cosentino S, Lukjancenko O, Saputra D, Rasmussen S, Hasman H, *et al*. (2014). Benchmarking of methods for genomic taxonomy. J Clin Microbiol 52(5):1529-1539. DOI:10.1128/JCM.02981-13.

Lee HJ, Orlovich DA, Tagg JR, Fawcett JP (2009). Detection and Specific Enumeration of Multi-Strain Probiotics in the Lumen Contents and Mucus Layers of the Rat Intestine After Oral Administration. Probiotics Antimicrob Proteins 1(2):113-120. DOI:10.1007/s12602-009-9019-6.

Lee HJ, Zhang H, Orlovich DA, Fawcett JP (2012). The influence of probiotic treatment on sulfasalazine metabolism in rat. Xenobiotica 42(8):791-797. DOI:10.3109/00498254.2012.660508.

Lee HJ, Waller RD, Stebbings S, Highton J. Orlovich DA, Schmierer D, Fawcett JP (2010). The effects of an orally administered probiotic on sulfasalazine metabolism in individuals with rheumatoid arthritis: a preliminary study. Int J Rheum Dis 13(1):48-54. DOI: 10.1111/j.1756-185X.2009.01449.x.

Leser TD, Mølbak L (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. Environ Microbiol 11(9):2194-2206. DOI:10.1111/j.1462-2920.2009.01941.x.

Martín R, Langa L, Reviriego C, Jiménez E, Marín ML, Olivares M, *et al*. (2004). Commensal microflora of human milk new perspectives for food bacteriotherapy. Trends Food Sci Technol 15(3&4):121-127.

Martín R, Heilig HG, Zoetendal EG, Jiménez E, Fernández L, Smidt H, *et al*. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. Res Microbiol 158(1):31-37.

Mogensen G, Salminen S, O'Brien J, Ouwehand A, Holzapfel WH, Shortt C, *et al*. (2002). Inventory of Microorganisms with a documented history of use in food. Bull IDF 377:10-19.

Moore AM, Patel S, Forsberg KJ, Wang B, Bentley G, Razia Y, *et al*. (2013). Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. PLoS One 8(11):e78822. DOI:10.1371/journal.pone.0078822.

Mora D, Arioli S (2014). Microbial urease in health and disease. PLoS Pathog 10(12):e1004472 [4pp]. DOI:10.1371/journal.ppat.1004472.

OECD (1998). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, no. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (2008). Repeated dose 28-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 407) [Adopted: 3 Oct 2008]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en.

Pariza MW, Gillies KO, Kraak-Ripple SF, Leyer G, Smith AB (2015). Determining the safety of microbial cultures for consumption by humans and animals. Regul Toxicol Pharmacol 73(1):164-171. DOI:10.1016/j.yrtph.2015.07.003.

Park H-K, Shim S-S, Kim S-Y, Park J-H, Park S-E, Kim H-J, *et al*. (2005). Molecular analysis of colonized bacteria in a human newborn infant gut. J Microbiol 43(4):345-353.

Patras KA, Wescombe PA, Rösler B, Hale JD, Tagg JR, Doran KS (2015). *Streptococcus salivarius* K12 Limits Group B Streptococcus Vaginal Colonization. Infect Immun 83(9):3438-3444. DOI:10.1128/IAI.00409-15.

Pehrsson EC, Tsukayama P, Patel S, Mejía-Bautista M, Sosa-Soto G, Navarrete KM, *et al*. (2016). Interconnected microbiomes and resistomes in low-income human habitats. Nature 533(7602):212-216. DOI:10.1038/nature17672.

Pešić-Mikulec D, Jovanović L (2005). Microbiological study of fresh white cheese (a Serbian craft variety). App Ecol Environ Res 4(1):129-134.

Pombert JF, Sistek V, Boissinot M, Frenette M (2009). Evolutionary relationships among salivarius streptococci as inferred from multilocus phylogenies based on 16S rRNA-encoding, recA, secA, and secY gene sequences. BMC Microbiol 9:232 [9pp]. DOI:10.1186/1471-2180-9-232.

Porter SR, Scully C, Hegarty AM (2004). An update of the etiology and management of xerostomia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 97(1):28-46.

Power DA, Burton JP, Chilcott CN, Dawes PJ, Tagg JR (2008). Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral probiotic *Streptococcus salivarius* K12. Eur J Clin Microbiol Infect Dis 27(12):1261-1263.

Scariya L, Nagarathna DV, Varghese M (2015). Probiotics in periodontal therapy. Int J Pharm Bio Sci 6(1):P242-P250.

Schleifer KH, Ehrmann M, Krusch U, Neve H (1991). Revival of the species Streptococcus thermophilus (ex Orla-Jensen, 1919) nom. rev. Syst Appl Microbiol 14:386-388. Cited In: Pombert *et al.*, 2009 [Ref. #5].

Seemann T (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14):2068-2069. DOI:10.1093/bioinformatics/btu153.

Shane AL, Cabana MD, Vidry S, Merenstein D, Hummelen R, Ellis CL, *et al*. (2010). Guide to designing, conducting, publishing and communicating results of clinical studies involving probiotic applications in human participants. Gut Microbes 1(4):243-253.

Sommer MOA, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. Science 325(5944):1128-1131. DOI:10.1126/science.1176950.

Sundin OH, Mendoza-Ladd A, Zeng M, Diaz-Arévalo D, Morales E, Fagan BM, *et al*. (2017). The human jejunum has an endogenous microbiota that differs from those in the oral cavity and colon. BMC Microbiol 17(1):160 [17pp]. DOI:10.1186/s12866-017-1059-6.

Tagg JR (2004). Prevention of streptococcal pharyngitis by anti-*Streptococcus pyogenes* bacteriocin-like inhibitory substances (BLIS) produced by *Streptococcus salivarius*. Indian J Med Res 119(Suppl.):13-16.

Tagg JR, Bannister LV (1979). 'Fingerprinting' beta-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. J Med Microbiol 12(4):397-411.

Tanzer JM, Kurasz AB, Clive J (1985). Competitive displacement of mutans streptococci and inhibition of tooth decay by *Streptococcus salivarius* TOVE-R. Infect Immun 48(1):44-50.

U.S. FDA (1995). Food labeling: Health claims; Sugar alcohols and dental caries (Final rule - 21 CFR Part 101 - Docket No. 95P–0003); proposed rule. Fed Regist (US) 60(130):37507-37549.

U.S. FDA (2000). *Agency Response Letter GRAS Notice No. GRN 000045 [Trehalose, Westminister (CO): Hayashibara International Inc.].* Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=45 [Oct. 5, 2000].

U.S. FDA (2001). *Agency Response Letter GRAS Notice No. GRN 000065 [Nisin, Cranbury (NJ): Rhodia, Inc.]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=065 [Apr. 20, 2001].

U.S. FDA (2011). *EAFUS: A Food Additive Database ["Everything" Added to Food in the United States]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=eafusListing [Page Last Updated: 05/18/2011].

U.S. FDA (2016). *Agency Response Letter GRAS Notice No. GRN 000591 [Streptococcus salivarius K12, Dunedin, New Zealand: BLIS Technologies Ltd.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=591 [Jan. 25, 2016].

U.S. FDA (2017). U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR [current to August 8, 2017].

Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
101—Food Labeling	101.12	Reference amounts customarily consumed per eating occasion
	101.80	Health claims: dietary noncariogenic carbohydrate sweeteners and dental caries
131—Food for Human Consumption	131.125	Nonfat dry milk
172—Food additives permitted for direct addition to food for human consumption	172.840	Polysorbate 80
182—Substances generally recognized as safe	182.1057	Hydrochloric acid
184—Direct food substances affirmed as generally recognized as safe	184.1135	Ammonium bicarbonate
	184.1138	Ammonium chloride
	184.1139	Ammonium hydroxide
	184.1140	Ammonium citrate, dibasic
	184.1141a	Ammonium phosphate, monobasic
	184.1141b	Ammonium phosphate, dibasic

Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
	184.1150	Bacterially-derived protease enzyme preparation
	184.1444	Maltodextrin
	184.1736	Sodium bicarbonate
	184.1854	Sucrose
	184.1983	Bakers yeast extract

Upton M, Tagg JR, Wescombe P, Jenkinson HF (2001). Intra- and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. J Bacteriol 183(13):3931-3938.

USDA (2009). *What We Eat In America: National Health and Nutrition Examination Survey (NHANES): 2003-2004, 2005-2006*. Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793.

Van den Bogert B, Boekhorst J, Herrmann R, Smid EJ, Zoetendal EG, Kleerebezem M (2013a). Comparative genomics analysis of Streptococcus isolates from the human small intestine reveals their adaptation to a highly dynamic ecosystem. PLoS One 8(12):e83418 [14pp]. DOI:10.1371/journal.pone.0083418.

van den Bogert B, Erkus O, Boekhorst J, de Goffau M, Smid EJ, Zoetendal EG, *et al*. (2013b). Diversity of human small intestinal Streptococcus and Veillonella populations. FEMS Microbiol Ecol 85(2):376-88. DOI:10.1111/1574-6941.12127.

Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I, Romond M-B, *et al*. (2008). Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci Technol 19(2):102-114.

Wang M, Ahrné S, Jeppsson B, Molin G (2005). Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol Ecol 54(2):219-231.

Watanabe S, Ohnishi M, Imai K, Kawano E, Igarashi S (1995). Estimation of the total saliva volume produced per day in five-year-old children. Arch Oral Biol 40(8):781-782.

Wescombe PA, Upton M, Dierksen KP, Ragland NL, Sivabalan S, Wirawan RE, *et al*. (2006). Production of the lantibiotic salivaricin A and its variants by oral streptococci and use of a specific induction assay to detect their presence in human saliva. Appl Environ Microbiol 72(2):1459-1466.

Wescombe PA, Upton M, Renault P, Wirawan RE, Power D, Burton JP, *et al*. (2011). Salivaricin 9, a new lantibiotic produced by Streptococcus salivarius. Microbiology 157(5):1290-1299. DOI:10.1099/mic.0.044719-0.

WHO/FAO (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Rome, Italy: World Health Organization (WHO) / Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/3/a-a1554e.pdf.

Wilson M, Martin R, Walk ST, Young C, Grossman S, McKean EL, *et al*. (2012). Clinical and laboratory features of *Streptococcus salivarius* meningitis: a case report and literature review. Clin Med Res 10(1):15-25. DOI:10.3121/cmr.2011.1001.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, *et al*. (2012). Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67(11):2640-2644. DOI:10.1093/jac/dks261.

Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, *et al*. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J. 6(7):1415-1426. DOI:10.1038/ismej.2011.212.

Zorrilla-Vaca A, Healy RJ, Rivera-Lara L, Grant MC, Maragakis LL, Escandón-Vargas K, *et al*. (2018). Epidemiology of septic meningitis associated with neuraxial anesthesia: a historical review and meta-analysis. Minerva Anestesiol 84(3):363-377 . DOI:10.23736/S0375-9393.17.11920-6.

From: Brian Watson
To: Zhu, Jianmei
Cc: John Hale
Subject: FW: Questions for GRN807
Date: Tuesday, November 27, 2018 3:24:28 AM
Attachments: COA_18-0293003-01315100-C.pdf

Dear Dr Zhu,
Please see below our response to your questions. Please feel free to contact me if you have additional questions.

Regards,
Brian

_____

Question 1.
You did not comment on the production of biogenic amines by M18. Are there data about the production of biogenic amines by M18 in vivo or have you checked for the decarboxylase genes commonly associated with bioamine production in the genome sequence of M18? If not, please explain why you would not consider that relevant to the safety of the intended use.

We had not considered running specific tests for biogenic amines based on the fact this is not common in S. salivarius bacteria, and has not been signalled to us with our S. salvivarius K12 GRAS application. A search of the S. salivarius M18 genome (https://jb.asm.org/content/193/22/6402) identified 5 genes with the search term "decarboxylase genes" listed in the below table, but none with "Bioamine production"

Found in S. salivarius M18 genome	Function/ end product
EGX31112.1 417 diaminopimelate decarboxylase diaminopimelate decarboxylase [Streptococcus salivarius M18] 345527804 AGBV01P000212 EGX31113.1 180 transcriptional regulator, TetR/AcrR family protein transcriptional regulator, TetR/AcrR family protein [Streptococcus salivarius M18] 345527805 AGBV01P000213	L-lysine
EGX29816.1 181 phosphopantothenoylcysteine decarboxylase phosphopantothenoylcysteine decarboxylase [Streptococcus salivarius M18] 345526505 AGBV01P000754	pantotheine 4'-phosphate
EGX29898.1 239 alpha-acetolactate decarboxylase alpha-acetolactate decarboxylase [Streptococcus salivarius M18] 345526587	acetoin

AGBV01P000836	
EGX29946.1 231 orotidine 5'-phosphate decarboxylase orotidine 5'-phosphate decarboxylase [Streptococcus salivarius M18] 345526635 AGBV01P000884	uridine monophosphate (UMP)
EGX30431.1 314 diphosphomevalonate decarboxylase diphosphomevalonate decarboxylase [Streptococcus salivarius M18] 345527120 AGBV01P001369	ADP + phosphate + isopentenyl diphosphate

Question 2.

On Page 16, you stated that skim milk powder is used in the manufacturing of M18. Milk is one of the 8 major allergens in the US. Please clarify whether there is any allergen in the final product. If not, please provide a statement to confirm that.

Testing of BLIS M18 raw ingredient has been carried out by an independent testing authority. Their results (attached) found

- Beta galacto globulin (milk sugar) >1 ppm (limits of detection are NT-BLG-01 01 LOR 0.1 ppm)
- Casein (Milk protein): 3.4 ppm (Limits of detection are NT-MILK-01 01 LOR 1 ppm)

Therefore while the levels of milk allergen are low, it cannot be considered dairy free by this test.

AsureQuality Limited | 131 Boundary Road | Blockhouse Bay | Auckland 0600 | New Zealand
PO Box 41 | Auckland 1140 | New Zealand
t. 00 64 9 626 8200 | e. VLabAuckland@asurequality.com | w. www.asurequality.com
Global Experts in Food Assurance

Certificate of Analysis

Final Report

Vidya Kulkarni
BLIS Technologies Laboratory
81 Glasgow Street
Dunedin 9012
New Zealand

PO Number: G 556651

Report Issued: 23-Nov-2018 **AsureQuality Reference: 18-293003** **Sample(s) Received:** 23-Nov-2018 08:45

Results

The tests were performed on the samples as received.

Customer Sample Name: M1845 **AsureQuality ID:** 18-293003-1

Batch/Lot No.: M1845 **Sample Condition**: Acceptable

Test	Result	Unit	Method Reference
Beta-Lacto Globulin	>1.00	ppm	ELISA Systems
Milk Casein	3.4	ppm	ELISA Systems

Analysis Summary

Auckland Laboratory

Analysis	Method	Accreditation	Authorised by
Beta lactoglobulin Allergen			
NT-BLG-01, 01-DEFAULT	ELISA Systems	IANZ	Tim Hudson
Milk Casein Allergen			
NT-MILK01, 01-DEFAULT	ELISA Systems	IANZ	Tim Hudson

Tim Hudson
Laboratory Analyst

Accreditation

IANZ
ACCREDITED LABORATORY

Appendix A
Certificates of Analysis

Certificate of Analysis

Streptococcus salivarius BLIS M18

Strain:	*Streptococcus salivarius* M18
Batch No:	13.02
Date of Production:	20 December 2013
Expiry Date:	20 December 2016

Analysis	Result	Specification	Method
ACTIVE			
S. salivarius M18	> 1 x 10^{11} cfu/g	NLT 1 x 10^{11} cfu/g	BLIS Technologies Ltd SOP PO11
MICROBIAL			
Coliforms	ND/g	ND/g	Compendium 4th Edn 2001 (mod)
E. coli	ND/g	ND/g	Compendium 4th Edn 2001 (mod)
Salmonella	ND/25 g	ND/25 g	ISO 6579:2002 (E)
Mesophilic Aerobic Spores	< 10 cfu/g	NMT 200 cfu/g	Compendium 4th Edn 2001
Staphylococcus aureus	ND/g	ND/g	ISO 6888-3:2003
Moulds	<10 cfu/g	NMT 50 cfu/g	BP 2010
Yeasts	<10 cfu/g	NMT 50 cfu/g	BP 2010
PHYSICAL CHARACTERISTICS			
Water Activity (a_w)	<0.2	<0.25	AquaLab Operator's Manual
Particle Size	d(0.9): 373 µm	d(0.9)<500 µm	Mastersizer 2000*
Arsenic	< 0.05 ppm	NMT 1 ppm	ICP-MS (APHA3125B)
Lead	< 0.05 ppm	NMT 0.5 ppm	ICP-MS (APHA3125B)
Mercury	< 0.05 ppm	NMT 0.15 ppm	ICP-MS (APHA3125B)
Cadmium	< 0.02 ppm	NMT 0.2 ppm	ICP-MS (APHA3125B)

Tests are performed at external accredited laboratories.
* Manufacturers internal test

Key to Terms: cfu: Colony Forming Unit ND: Not Detected NLT: Not Less Than NMT: Not More Than

Streptococcus salivarius M18 freeze-dried live culture complies with the Product Specification TM18.2.3v2US and is expected to remain in specification for a minimum of 36 months if maintained under recommended storage and handling conditions.

Store between 2 to 8°C in dry conditions away from sunlight. DO NOT FREEZE.

Vidya Kulkarni, Quality Assurance and Quality Control. Date of Approval:20 December 2013

BLIS Technologies Ltd, Level 1, Centre for Innovation, 87 St David St
PO Box 56, Dunedin, New Zealand
Phone: +64 3 4793061 Fax: +64 3 4798954 Email: info@blis.co.nz

Document Number: TM18I.4.3v2US
Date of Issue: April 2014
Replaces: TM18I.4.3v1US

Certificate of Analysis

Streptococcus salivarius BLIS M18

BLIS Technologies

Strain:	*Streptococcus salivarius* M18
Batch No:	16.06
Date of Production:	25 April 2016
Expiry Date:	25 April 2019

Analysis	Result	Specification	Method
ACTIVE			
S. salivarius M18	> 1 X 10^{11} cfu/g	NLT 1 x 10^{11} cfu/g	BUS Technologies Ltd SOP PO11
MICROBIAL			
Coliforms	ND/g	ND/g	Compendium 5th Edn 2015 (mod)
E coli	ND/g	ND/g	Compendium 5th Edn 2015 (mod)
Salmonella	ND/25g	ND/25 g	ISO 6579:2002 (E)
Mesophilic Aerobic Spores	< 10 cfu/g	NMT 200 cfu/g	Compendium 5th Edn 2015
Coagulase producing *Staphylococcus*	ND/g	ND/g	ISO 6888-3:2003
Moulds	<10 cfu/g	NMT 50 cfu/g	British pharmacopoeia
Yeasts	<10 cfu/g	NMT 50 cfu/g	British pharmacopoeia
PHYSICAL CHARACTERISTICS			
Water Activity (aw)	<0.2	<0.25	AquaLab Operator's Manual
Particle Size	d (0.9): 397 µm	D (0.9) <500 µm	Mastersizer 2000*
Arsenic	<0.05 ppm	NMT 1 ppm	In-house digestion/I CP-MS(APHA3125B)
Lead	<0.05 ppm	NMT 0.5 ppm	In-house digestion/ICP-MS(APHA3125B)
Mercury	<0.05 ppm	NMT 0.15 ppm	In-house digestion/ICP-MS(APHA3125B)
Cadmium	<0.02 ppm	NMT 0.2 ppm	In-house digestion/ICP-MS(APHA3125B)

Tests are performed at external accredited laboratories.
* Manufacturers internal test

Key to Terms: cfu: Colony Forming Unit ND: Not Detected NLT: Not Less Than NMT: Not More Than

Streptococcus salivarius M18 freeze-dried live culture complies with the Product Specification TM181.2.3v3US and is expected to remain in specification for a minimum of 36 months if maintained under recommended storage and handling conditions.

Store between 2 to 8°C (36 to 46°F) in dry conditions away from sunlight. DO NOT FREEZE.
Product is expected to remain stable if exposed up to 18°C (65°F) for up to 48 hours and then returned to stipulated storage conditions.

Vidya Kulkarni, Quality d Quality Control Date of Approval: 21 June 2016

Blis Technologies Limited, 10 Birch Street,
P O Box 5804, Dunedin 9016, New Zealand
Telephone: +64 3 4741338 Email: info@blis.co.nz

Document Number: TM181.4.3v3US
Issue Date: February 2016
Replaces: TM181.4.3v2US

Certificate of Analysis

Streptococcus salivarius BLIS M18

BLIS Technologies

Strain:	*Streptococcus salivarius* M18
Batch No:	16.10
Date of Production:	22 July 2016
Expiry Date:	22 July 2019

Analysis	Result	Specification	Method
ACTIVE			
S. salivarius M18	> 1 X 10^{11} cfu/g	NLT 1 x 10^{11} cfu/g	BLIS Technologies Ltd SOP PO11
MICROBIAL			
Coliforms	ND/g	ND/g	Compendium 5th Edn 2015 (mod)
E.coli	ND/g	ND/g	Compendium 5th Edn 2015 (mod)
Salmonella	ND/25g	ND/25 g	ISO 6579:2002 (E)
Mesophilic Aerobic Spores	< 10 cfu/g	NMT 200 cfu/g	Compendium 5th Edn 2015
Coagulase producing *Staphylococcus*	ND/g	ND/g	ISO 6888-3:2003
Moulds	<10 cfu/g	NMT 50 cfu/g	British pharmacopoeia
Yeasts	<10 cfu/g	NMT 50 cfu/g	British pharmacopoeia
PHYSICAL CHARACTERISTICS			
Water Activity (aw)	<0.2	<0.25	AquaLab Operator's Manual••
Particle Size	d (0.9): 367 µm	D (0.9) <500 µm	Mastersizer 2000•
Arsenic	<0.05 ppm	NMT 1 ppm	In-house digestion/ICP-MS(APHA3125B)
Lead	<0.05 ppm	NMT 0.5 ppm	In-house digestion/ICP-MS(APHA3125B)
Mercury	<0.05 ppm	NMT 0.15 ppm	In-house digestion/ICP-MS(APHA3125B)
Cadmium	<0.02 ppm	NMT 0.2 ppm	In-house digestion/ICP-MS(APHA3125B)

Tests are performed at external accredited laboratories.

* Manufacturers internal test; ** Blis internal test

Key to Terms: cfu: Colony Forming Unit ND: Not Detected NLT: Not Less Than NMT: Not More Than.

Streptococcus salivarius M18 freeze-dried live culture complies with the Product Specification TM181.2.1 v7 and is expected to remain in specification for a minimum of 36 months if maintained under recommended storage and handling conditions.

Store between 2 to 8°C in dry conditions away from sunlight. DO NOT FREEZE.

Vidya Kulkarni, Quality Assurance and Quality Control

Date of Approval: 1 August 2016

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Document Number: TM18I.4.1v6
Issue Date: March 2014
Replaces: TM18I.4.1v5

Appendix B
FSANZ Not 'Novel' Opinion for M18

FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

55 Blackall St., Barton
ACT 2600 Australia
PO Box 7186
Canberra BC ACT 2610
Australia
Tel+ 61 2 6271 2222
Fax +61 2 6271 2278
www.foodstandards.gov.au

Level 6, 108 The Terrace
Wellington
PO Box 10 559
Wellington
Tel + 64 4 978 5630
Fax+ 64 4 473 9855
www.foodstandards.govt.nz

Dr Barry Richardson
CEO
BUS Technologies Ltd
Centre for Innovation
University of Otago
PO Box 56
DUNEDIN 9054
NEW ZEALAND

Dear Dr Richardson

Thank you for your enquiry of 22 November 2012 regarding *Streptococcus salivarius* M18 as a potential novel food. You enquired whether in the view of the Advisory Committee on Novel Foods (the Committee) [1] an application to amend Standard 1.5.1 - Novel Foods of the *Australia New Zealand Food Standards Code* (the Code) should be made.

Standard 1.5.1 of the Code requires FSANZ to conduct a pre-market safety assessment of those non-traditional foods that are deemed to be novel according to the definitions in the Standard. The definitions of 'non-traditional food' and 'novel food' are provided in the Attachment to this letter.

The Committee discussed your enquiry at its meeting on 12 December 2012 and used the Guidance Tool to assist in forming a view. The Guidance Tool is divided into Part 1 - Determining whether a food is non-traditional or not; and Part 2 - Determining whether an assessment of public health and safety considerations is required for a non-traditional food.

The Committee has formed the view that *Streptococcus salivarius* M18 does not meet the definition of 'non-traditional food' on the basis that this food has a history of human consumption in Australia or New Zealand (part 1 of the guidance tool).

Streptococcus salivarius M18 therefore, is also considered by the Committee to be not 'novel' in Australia and New Zealand, since a food may only be considered to be 'novel' in accordance with Standard 1.5.1 if it is firstly considered to be 'non-traditional'.

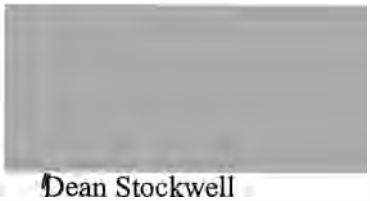
[1] The Advisory Committee on Novel Foods comprises representatives from Australian State and Territory jurisdictions, the Australian Quarantine Inspection Service, the New Zealand Food Safety Authority and Food Standards Australia New Zealand. The Committee provides recommendations to the General Manager/ Food Standards (Wellington) as to whether particular foods meet the definitions of 'non-traditional food' and 'novel food' in Standard 1.5.1 of the Code.

It is the responsibility of manufacturers, suppliers or importers to ensure products comply with the requirements of the Code. FSANZ is not responsible for enforcing the requirements of the Code. Enforcement of the Code is the responsibility of the Commonwealth, State, Territory and New Zealand Governments. Accordingly, the interpretation and application of Standard 1.5.1, including decisions about the novelty of a food or food ingredient, is ultimately the responsibility of those jurisdictions. Therefore while the Committee may express a view about whether or not *Streptococcus salivarius* M18 meets the definition of a novel food for the purposes of Standard 1.5.1, it is ultimately a decision for the relevant enforcement authority.

You should also note that Standard 1.1A.2 - Transitional Standard - Health Claims of the Code prohibits the making of health claims on food labels or advertisements for food, including claims for therapeutic or prophylactic action.

If you wish to discuss this matter further, please contact Mr Jonathon Kite on + 61 2 6271 2646 or jonathon.kite@foodstandards.gov.au.

Yours sincerely



Dean Stockwell
General Manager
Food Standards (Wellington)

)../ December 2012

cc. Dr John Hale (john.hale@blis.co.nz)

ATTACHMENT

non-traditional food means -

(a) a food that does not have a history of human consumption in Australia or New Zealand; or

(b) a substance derived from a food, where that substance does not have a history of human consumption in Australia or New Zealand other than as a component of that food; or

(c) any other substance, where that substance, or the source from which it is derived, does not have a history of human consumption as a food in Australia or New Zealand.

novel food means a non-traditional food and the food requires an assessment of the public health and safety considerations having regard to -

(a) the potential for adverse effects in humans; or
(b) the composition or structure of the food; or
(c) the process by which the food has been prepared; or
(d) the source from which it is derived; or
(e) patterns and levels of consumption of the food; or
(f) any other relevant matters.

Appendix C
Bioinformatic Results for Pathogenicity

Table C1. PATRIC 3.5.7 BLAST Alignments of *S. salivarius* M18 Annotated Genome Against Virulence Genes from the Victors Database

Source Organism	Product	PubMed	Query Coverage	Identity	E-value
Streptococcus pneumoniae TIGR4	Phosphate transport system permease protein PstC (TC 3.A.1.7.1)	12207705	97	82	1e-136
Streptococcus pneumoniae D39	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	15664911	99	94	1e-101
Streptococcus pneumoniae TIGR4	Ribonuclease J1 (endonuclease and 5' exonuclease)	12207705	98	90	1e-296
Streptococcus pneumoniae D39	Superoxide dismutase [Mn] (EC 1.15.1.1)	10768978	100	87	1e-101
Streptococcus pneumoniae Hungary19A-6	Glutamine synthetase type I (EC 6.3.1.2)	16787930	100	87	1e-237
Streptococcus pneumoniae TIGR4	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	12207705	100	85	1e-177
Streptococcus pneumoniae R6	Manganese ABC transporter, periplasmic-binding protein SitA	8945574	87	86	1e-140
Streptococcus pneumoniae TIGR4	Acetolactate synthase large subunit (EC 2.2.1.6)	12207705	100	87	1e-294
Streptococcus pneumoniae TIGR4	ClpE-like protein	12207705	90	91	0.0
Streptococcus pyogenes M1 GAS	Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	11679068	100	87	1e-146
Streptococcus pneumoniae D39	S-ribosylhomocysteine lyase (EC 4.4.1.21) @ Autoinducer-2 production protein LuxS	15102809	98	82	2e-72
Streptococcus agalactiae A909	Translation elongation factor LepA	10998175	99	97	0.0
Streptococcus pneumoniae D39	Phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)	11359563	94	87	5e-85
Streptococcus pneumoniae TIGR4	D-alanine--poly(phosphoribitol) ligase subunit 1 (EC 6.1.1.13)	12207705	100	87	1e-265
Streptococcus pneumoniae D39	Adenylosuccinate lyase (EC 4.3.2.2) @ SAICAR lyase (EC 4.3.2.2)	11359563	100	96	1e-245
Streptococcus pneumoniae TIGR4	5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20)	12207705	97	88	1e-147
Streptococcus pyogenes MGAS315	Lanthionine biosynthesis protein LanM	19223485	69	96	1e-275
Streptococcus pyogenes M1 GAS	tRNA-5-carboxymethylaminomethyl-2-thiouridine(34) synthesis protein MnmG	18426891	99	90	0.0
Streptococcus pneumoniae TIGR4	Ribosyl nicotinamide transporter, PnuC-like	12207705	97	89	1e-74
Streptococcus pyogenes MGAS5005	Catabolite control protein A	20333240	100	83	1e-159
Streptococcus agalactiae 2603V/R	Two component system response regulator CiaR	19114476	98	87	1e-109
Streptococcus pneumoniae TIGR4	Glycerol dehydrogenase (EC 1.1.1.6)	12207705	81	85	1e-147

Table C1. PATRIC 3.5.7 BLAST Alignments of *S. salivarius* M18 Annotated Genome Against Virulence Genes from the Victors Database

Source Organism	Product	PubMed	Query Coverage	Identity	E-value
Streptococcus pneumoniae TIGR4	Alcohol dehydrogenase (EC 1.1.1.1)	12207705	100	86	1e-177
Streptococcus pneumoniae TIGR4	Glycine betaine ABC transport system, ATP-binding protein OpuAA (EC 3.6.3.32)	12207705	99	88	1e-120
Streptococcus pneumoniae TIGR4	Phosphate transport system permease protein PstA (TC 3.A.1.7.1)	12207705	100	86	1e-144
Streptococcus pneumoniae D39	Pyruvate oxidase (EC 1.2.3.3)	8820650	97	95	5e-16
Streptococcus pneumoniae G54	Phosphoribosylformylglycinamidine synthase, synthetase subunit (EC 6.3.5.3) / Phosphoribosylformylglycinamidine synthase, glutamine amidotransferase subunit (EC 6.3.5.3)	11359563	100	92	0.0
Streptococcus pneumoniae TIGR4	Rhodanese domain protein UPF0176, Firmicutes subgroup	12207705	98	92	1e-184
Streptococcus pneumoniae D39	Pyruvate oxidase (EC 1.2.3.3)	8820650	100	95	4e-13
Streptococcus pneumoniae TIGR4	CTP synthase (EC 6.3.4.2)	12207705	99	91	1e-286
Streptococcus pneumoniae TIGR4	Oligopeptide transport system permease protein OppC (TC 3.A.1.5.1)	12207705	99	82	1e-151
Streptococcus pneumoniae TIGR4	IMP cyclohydrolase (EC 3.5.4.10) / Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3)	12207705	100	95	1e-285
Streptococcus pyogenes M1 GAS	Ribonuclease Y	20385762	100	82	1e-253
Streptococcus pneumoniae TIGR4	Xanthine phosphoribosyltransferase (EC 2.4.2.22)	12207705	99	87	2e-89
Streptococcus pneumoniae R6	ABC transporter, permease protein (cluster 3, basic aa/glutamine/opines)	18174343	100	87	1e-109
Streptococcus pneumoniae TIGR4	Two-component transcriptional response regulator, LuxR family	12207705	99	84	1e-110
Streptococcus pneumoniae TIGR4	Aspartate--ammonia ligase (EC 6.3.1.1)	12207705	100	88	1e-172
Streptococcus pneumoniae D39	Aminopeptidase C (EC 3.4.22.40)	11359563	97	82	1e-214
Streptococcus pneumoniae TIGR4	Phosphopentomutase (EC 5.4.2.7)	12207705	99	92	1e-219
Streptococcus pneumoniae R6	ABC transporter, permease protein (cluster 3, basic aa/glutamine/opines)	18174343	96	88	1e-108
Streptococcus pneumoniae TIGR4	Leucyl-tRNA synthetase (EC 6.1.1.4)	12207705	100	96	0.0
Streptococcus agalactiae A909	GMP synthase [glutamine-hydrolyzing], amidotransferase subunit (EC 6.3.5.2) / GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)	10998175	100	93	1e-288
Streptococcus agalactiae A909	DNA-directed RNA polymerase delta subunit (EC 2.7.7.6)	16513739	90	81	8e-81

Table C1. PATRIC 3.5.7 BLAST Alignments of *S. salivarius* M18 Annotated Genome Against Virulence Genes from the Victors Database

Source Organism	Product	PubMed	Query Coverage	Identity	E-value
Streptococcus pneumoniae TIGR4	Pyruvate formate-lyase activating enzyme (EC 1.97.1.4)	12207705	95	81	1e-119
Streptococcus pneumoniae TIGR4	C5a peptidase precursor (EC 3.4.21.-)	12207705	92	94	0.0
Streptococcus pneumoniae TIGR4	DNA polymerase III polC-type (EC 2.7.7.7)	12207705	84	81	0.0
Streptococcus pneumoniae TIGR4	Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)	12207705	96	84	1e-119

Appendix D
Expert Panel Consensus Statement

Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Use of *Streptococcus salivarius* M18 in Conventional Food Products

01 May 2018

INTRODUCTION

Blis Technologies Ltd. (BLIS) intends to market *Streptococcus salivarius* M18 (*S. salivarius* M18) freeze-dried powder in the United States (U.S.) for use as a food ingredient in conventional food and beverage products across multiple food categories [*i.e.,* baby, infant, and toddler foods (excluding infant formula); baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogues; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices; soft candy; sweet sauces, toppings, and syrups; and medical foods] at a level of 20 mg per serving [providing a minimum of $1x10^9$ colony-forming units (CFU)/serving].

BLIS convened a panel ("the Expert Panel") of independent scientists, qualified by their scientific training and relevant national and international experience, to conduct a critical and comprehensive evaluation of the available pertinent data and information on *S. salivarius* M18 freeze-dried powder, and determine whether the aforementioned uses of the ingredient are Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of Dr. Joseph Borzelleca, Ph.D., (Virginia Commonwealth University School of Medicine), Dr. Robert Nicolosi, Ph.D., (University of Massachusetts Lowell), and Dr. Michael Pariza, Ph.D. (University of Wisconsin). For the purposes of the Expert Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined by the U.S. Food and Drug Administration (FDA) in 21 CFR 170.3(i) (U.S. FDA, 2017).

The Expert Panel, independently and collectively, critically evaluated a dossier which included a summary of the scientific information on *S. salivarius* M18 prepared from a comprehensive search of the scientific literature, including both favorable and unfavorable data and information, as well as details pertaining to the method of manufacture and product specifications; supporting analytical data; intended conditions of use of freeze-dried *S. salivarius* M18 powder in food; estimated exposure under the proposed food-uses; and a comprehensive assessment of the available scientific literature pertaining to the safety of the microorganism. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent, critical evaluation of such data and information, the Expert Panel convened *via* teleconference on 01 May 2018, and unanimously concluded that *S. salivarius* M18, meeting appropriate food-grade specifications as described in the supporting dossier [*Documentation Supporting the Generally Recognized as Safe (GRAS) Status of Streptococcus salivarius M18 for Use as an Ingredient in Conventional Food Products*], and manufactured according to current Good Manufacturing Practice (cGMP), is GRAS under the conditions of intended use described in Table A-1 based on scientific procedures. A summary of the basis for the Expert Panel's conclusion, excluding confidential data and information, is provided below.

CHARACTERIZATION OF *S. SALIVARIUS* M18

The ingredient that is the subject of this GRAS evaluation is *Streptococcus salivarius* M18 freeze-dried powder. *S. salivarius* M18 is a Gram-positive, non-hemolytic, non-spore forming cocci that was originally isolated from the oral cavity of a healthy adult human. The organism can be selectively cultivated on Mitis salivarius agar and phenotypic profiling of the organism has been conducted using conventional biochemical techniques, including the API 20 Strep and API 50 carbohydrate fermentation profile strip testing procedures. The genome (chromosomal and megaplasmid DNA) of the organism has been sequenced using shotgun sequencing methods (Heng *et al.,* 2011). Analysis of the whole genome of *S. salivarius* M18 using KmerFinder 2.0 against 16-mer sequences generated from 1,647 complete bacterial genomes downloaded from the National Center for Biotechnology Information database identified *S. salivarius* CCHSS3 and JIM8777 as species matches, confirming the identity of the M18 strain as *S. salivarius*. Strain M18 was shown to display a unique DNA fingerprint when analyzed by enterobacterial repetitive intergenic consensus (ERIC)-polymerase chain reaction (PCR) molecular typing, or pulsed field gel electrophoresis (PFGE), and was therefore considered robustly characterized at the strain level to allow for quality control monitoring of the organism during manufacture, and for use in post-market surveillance.

S. salivarius M18 has been deposited in the American Type Culture Collection (ATCC) as ATCC BAA 2593 and the German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkuturen (DSMZ) GmbH] under the accession number DSMZ 14685. Master cultures and working cultures are maintained at BLIS and the Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand.

MANUFACTURING AND SPECIFICATIONS

The Expert Panel reviewed information provided by BLIS describing the chemistry and manufacturing of the *S. salivarius* M18 freeze-dried powder. The Expert Panel reviewed documentation supporting that *S. salivarius* M18 is fermented under cGMP using permitted and suitable fermentation processing-aids and understands that quality control methods are implemented throughout various stages of fermentation to ensure production of a pure culture which is absent of contaminating pathogens. Lyoprotectants, including trehalose, lactitol, and maltodextrin, are added to improve stability and viability of the freeze-dried organism. All these materials are currently permitted for use in food in the U.S. The Expert Panel noted that in accordance with the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004, milk must be declared on the label as a potential allergen due to the use of skim milk powder in the fermentation media (U.S. FDA, 2004).

Suitable food-grade specifications have been developed for *S. salivarius* M18 freeze-dried powder, ensuring the final food-grade product is of high purity and free of microbiological contaminants and heavy metals. Batch analyses of 3 non-consecutive lots of the ingredient in compliance with product specifications were provided to the Expert Panel. BLIS reported that *S. salivarius* M18 freeze-dried powder is stable for at least 36 months at 5 ± 3°C, which is the recommended storage temperature.

INTENDED USES AND CONSUMPTION ESTIMATES

BLIS intends to market *S. salivarius* M18 freeze-dried powder as an ingredient in the same food and beverage categories as *S. salivarius* K12 (see Table A-1), a similar ingredient that is GRAS for use as a food ingredient in the U.S. [GRAS Notice (GRN) 591; U.S. FDA, 2016]. Consumption estimates for the intake of *S. salivarius* K12 following introduction to the U.S. marketplace were based on the intended food-uses and

use-levels in conjunction with food consumption data included in the 2003-2004 and 2005-2006 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006, 2009; USDA, 2009) and are reported in Section IV.A.2 of GRN 591 (U.S. FDA, 2016). Since *S. salivarius* M18 freeze-dried powder is intended for use in the same foods and food categories as those defined for *S. salivarius* K12, estimated intakes of *S. salivarius* M18 are incorporated by reference to data presented in GRN 591 (U.S. FDA, 2016). Among users only, the mean intake of *S. salivarius* M18 by the total U.S. population from all proposed food-uses is estimated to be $9.8x10^9$ CFU/person/day. On an individual basis, the greatest mean all-user intake of *S. salivarius* M18 is predicted to be male teenagers at $1.2x10^{10}$ CFU/person/day. Foods containing *S. salivarius* M18 will be marketed in select foods products targeted to individuals seeking probiotic foods. Market experiences with similar types of health-based food ingredients (*e.g*., phytosterols) have demonstrated that heavy consumption by individuals consuming large numbers of food products to which *S. salivarius* M18 may be added is unlikely to occur (EFSA, 2008). The Expert Panel therefore considered the intake estimations to represent gross exaggerations of that expected following introduction of the strain to the U.S. marketplace.

DATA PERTAINING TO SAFETY

The Expert Panel reviewed a large body of safety data during their assessment of the safety of *S. salivarius* M18 freeze-dried powder; this information included both strain-specific information and relevant safety data on non-related strains of *S. salivarius* (*i.e., S. salivarius* K12), and is summarized briefly in the sections that follow.

History of Safe Consumption

The Expert Panel noted that strains of *S. salivarius* have a long history of use as starter cultures for the manufacture of cheese and yogurt. Strains of *S. salivarius* are natural residents of the human oral cavity and have been identified in infants within 2 days after birth. The daily consumption of commensal *S. salivarius* through natural ingestion of saliva in humans is estimated to range from $5x10^9$ to $5x10^{10}$ CFU/day. *S. salivarius* is one of the predominant bacterial species in human breast milk (Heikkilä and Saris, 2003; Martín *et al.,* 2004, 2007; Dalidowitz, 2005), and has been identified in the gastrointestinal tract of infants (Favier *et al.*, 2002; Park *et al.*, 2005). Consistent with its established history of safe use, *S. salivarius* K12 has GRAS status for use in a variety of food and beverage products at use-levels of up to $2x10^9$ CFU per serving (GRN 591) (U.S. FDA, 2016). Generally available discussions on the history of use, non-pathogenicity, and non-toxicogenicity of the *S. salivarius* species are incorporated by reference to GRN 591 (U.S. FDA, 2016). *S. salivarius* K12 has been cleared by Health Canada and the Australia New Zealand Food Safety Authority (FSANZ) and is a Listed Medicine by the Australian Therapeutics Goods Administration (TGA). The strain that is the subject of this evaluation, *S. salivarius* M18, has also been cleared by FSANZ for general food use based on the history of use of the species in food and corresponding exemption from the novel food regulations in Australia/New Zealand.

Persistence and Metabolic Fate

S. salivarius predominantly inhabits the dorsum of the tongue and the pharyngeal mucosa in humans (Gibbons and van Houte, 1975) and it is well established that *Streptococcus* sp., including *S. salivarius* isolates, form a dominant phylum throughout all gastrointestinal sites proximal to the terminal ileum (Wang *et al*., 2005; Booijink *et al*., 2007, 2010; Zoetendal *et al*., 2012; Sundin *et al*., 2017). Regions of the gastrointestinal tract proximal to the terminal ileum are environments where food components encounter bacteria. *Streptococcus* sp. exhibit very fast sugar transport and metabolism systems and are therefore well

adapted for survival in this environment where uptake of nutrients and gastrointestinal transit is rapid (Booijink *et al.*, 2010; Zoetendal *et al*., 2012). The rapid internalization and conversion of simple carbohydrates to support growth is a prominent strategy for microbial proliferation and microbiota maintenance in the small intestine and differentiates the microbiota of the small intestine with populations residing within the large intestine, which is largely absent of *Streptococcus* sp. (Wang *et al*., 2005; Sundin *et al*., 2017). The functional significance of microbiota populations present within the small intestine are unclear. Studies conducted using germ-free animals suggest that microbial populations indigenous to the small intestine can influence host gene expression including upregulation of nutrient transport genes and upregulation of genes with putative roles in mucosal barrier function and epithelial barrier integrity (Leser and Molbak, 2009).

There is no strain-specific information characterizing the gastrointestinal colonization of *S. salivarius* M18; however, the indigenous microflora profiles of most animals are intrinsically highly stable and resistant to colonization by exogenous microorganisms. Permanent lifelong colonization by ingested microorganisms is rare (WHO/FAO, 2009). The Expert Panel reviewed studies evaluating the oral persistence of *S. salivarius* M18 in adults and children following repeated ingestion of up to $1x10^9$ and $7.2x10^9$ CFU per day for 28 and 90 days, respectively (Burton *et al*., 2013a,b). These studies, which are described in greater detail below, support the aforementioned conclusion that long-term persistence of ingested M18 is not expected and that transient colonization of the oral cavity will not result in detectable population shifts of the indigenous microbial populations. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the cellular components (proteins, lipids, carbohydrates) used as a source of nutrients. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the faces.

Toxicological Studies

No strain specific studies examining the potential toxicological effects of *S. salivarius* M18 were conducted. Toxicology testing was conducted for *S. salivarius* K12 and was reported in GRN 591 (U.S. FDA, 2016), and included an acute study in rats (up to 5,000 mg/kg body weight or $8x10^{10}$ CFU), a 28-day repeated-dose toxicity study in rats (up to 5,000 mg/kg body weight/day), and genotoxicity testing using the Ames test (up to 5,000 µg/plate). *S. salivarius* K12 did not produce toxicological effects in these studies. As discussed in GRN 591, due to methodological limitations [*e.g.,* deviations from the Organisation for Economic Co-operation and Development (OECD) and Good Laboratory Practice (GLP) guidelines], derivation of a no-observed-adverse-effect level (NOAEL) for risk assessment purposes was not possible; however, the Expert Panel noted that there were no toxicological findings suggestive that the strain would be unsafe for human consumption. Findings from these studies corroborate the safety of *S. salivarius* M18.

Human Studies with *Streptococcus salivarius* M18

The oral colonization of *S. salivarius* M18 was evaluated in a randomized blinded clinical study, in which young adults (average age 19 years; gender not reported) consumed lozenges containing *S. salivarius* M18 (CFU per day: $1x10^6$, n=19; $1x10^7$, n=20, $1x10^8$, n=17, $1x10^9$, n=19) for 28 days (Burton *et al.,* 2013a). Saliva samples were collected at baseline and weekly thereafter to measure salivary levels of *S. salivarius,* including strain M18. Total mean salivary *S. salivarius* did not change from baseline following exposure to *S. salivarius* M18 for 28 days, indicating that exposure to *S. salivarius* M18 did not disrupt the indigenous oral microflora. *S. salivarius* M18 counts increased compared to baseline during the first week of exposure (Day 7) in a partially dose-dependent manner, suggesting that a proportion of the original *S. salivarius* population was replaced by strain M18. *S. salivarius* M18 remained elevated in all dose groups for the remainder of the study with a slight downward trend from Week 1. The percentage of subjects with

detectable levels of *S. salivarius* M18 in their saliva also increased with the dose, with the lowest dose group having a maximum of about 42% of subjects with *S. salivarius* M18 at Day 14, for example, whereas 100% of subjects had *S. salivarius* M18 in the highest dose group at Days 7 and 14. Levels of *S. salivarius* M18 were not measured after lozenge consumption ceased.

The effect of *S. salivarius* M18 on indices of dental health was evaluated in a randomized double-blind placebo-controlled clinical trial in healthy male and female school children (average age 8.9 years) with a history of dental caries (Burton *et al.,* 2013b). Children in the probiotic group consumed 2 lozenges per day each containing $3.6x10^9$ CFU *S. salivarius* M18 (n=40) for 3 months and the placebo group (n=43) received lozenges without probiotic. Saliva samples were collected at baseline, 1, 2, 3, and 7 months for the measurement of indicator microbes (*S. mutans, lactobacilli* and yeast) as well as *S. salivarius* (total count and strain M18). Monthly interviews were conducted with parents/guardians to determine if the children had experienced any adverse events. Four cases of adverse reactions were reported during the monthly questionnaires. Three of the self-reported events occurred in the *S. salivarius* M18 group and included a sore throat and 2 cases of chickenpox. One bleeding gum event occurred in the placebo group. None of the adverse events were of a serious nature and no subject left the trial as a result. The acquisition of a new *S. pyogenes* hemolytic colony during the treatment period was reported in 18% of subjects in the M18 group *vs*. 26% of the children in the placebo group (P>0.05). No differences between the treatment and placebo groups were reported for *S. mutans* carriage rates and gingival soft tissue health. Colonization of the M18 strain at the 3-month time-point was poor; however, 9 subjects had M18 populations comprising at least 5% of their total salivary *S. salivarius*. Findings from this study demonstrate that *S. salivarius* M18 is well tolerated at levels providing $7.2x10^9$ CFU/day for 3 months. At these ingestion levels, persistence of M18 was transient in most children and no detectable population shifts in these non-targeted microbes were reported.

A randomized controlled clinical study was conducted in a population of healthy male and female children (6 to 17 years) at high risk for dental caries to assess the safety and tolerability of oral tablets containing *S. salivarius* M18 and to evaluate effects on dental caries formation (Di Pierro *et al.,* 2015). Subjects were orally supplemented once a day with slowly dissolving tablets containing *S. salivarius* M18 (>$1x10^9$ CFU/tablet) (n=38) or a control group did not receive any treatment (n=38) for 90 days. Subjects were evaluated every 15 days by the study dentists for probiotic tolerability and dosing compliance and the occurrence of any side effects was reported by the subjects and/or their parents. Tolerability was reported as “very good” and “good” in 35 out of 38 subjects, and “acceptable” in the remaining 3 subjects. No treatment-related side effects were reported in any of the subjects supplemented with *S. salivarius* M18, and the authors concluded that *S. salivarius* M18 demonstrated a “very good” safety profile.

A randomized controlled study was conducted in healthy male and female adults (20 to 60 years) with moderate and severe gingivitis and moderate periodontitis (Scariya *et al.,* 2015). Subjects were instructed to consume 2 lozenges containing *S. salivarius* M18 (>$1x10^8$ CFU/lozenge; n=7/sex) twice daily (*i.e.,* once in the morning and in the evening) for 30 days. Subjects in the control group (n=7/sex) did not consume any lozenges. Several clinical parameters were assessed at baseline and at Days 15, 30, 45, and 60, including supragingival plaque, gingival index, bleeding on probing, and probing pocket depth. Safety parameters were not assessed in the study and no adverse reactions were reported by any of the subjects.

Several studies using *S. salivarius* K12 were conducted in adults, children, and infants, providing corroborative evidence for the safety of the intended use of *S. salivarius* M18 in food. One randomized, double-blind, placebo-controlled, parallel arm study specifically evaluated the safety of *S. salivarius* K12 in adult subjects (Burton *et al*., 2011). Consumption of up to $1.1x10^{10}$ CFU/day of *S. salivarius* K12 for 28 days was reported to be well-tolerated, and any reported adverse effects were either mild or were not related to

S. salivarius K12. The remaining clinical studies with *S. salivarius* K12 were not conducted to examine safety-related endpoints. The absence of reported adverse effects in these studies corroborates the safety of *S. salivarius*.

Assessment of Pathogenic and Toxigenic Potential

S. salivarius is a natural inhabitant of the oropharynx and the gastrointestinal tract, and in rare instances it can be an opportunistic pathogen in individuals with a serious underlying condition. Numerous case reports of infection related to *S. salivarius* have been reported in the literature, with iatrogenic cases of meningitis from improper surgical practice representing one of the most common causes of these opportunistic infections. This route of exposure is not relevant to food applications and no cases of spontaneous meningitis have been reported in otherwise healthy individuals. Clinical cases of opportunistic infection have been successfully treated with antibiotics and few instances of significant sequelae have been reported to result from *S. salivarius* infection (Wilson *et al.*, 2012). Case reports of opportunistic infections with *S. salivarius* are comparable to similar reports of opportunistic infections involving lactobacilli or bifidobacteria, including strains commonly used in fermented foodstuffs (Borriello *et al*., 2003).

S. salivarius does not contain any major virulence factors that have been described for pathogenic streptococci, and extensive genomic and bioinformatic analyses of various clinical isolates have not identified genetically controlled pathogenicity traits. For example, Delorme *et al.* (2007) evaluated the relationship between the commensal strains of *S. salivarius* and the strains of *S. salivarius* associated with invasive infections using sequence analyses and multi-locus sequence typing. The authors reported that the infection-associated strains could not be distinguished from the commensal strains, thus suggesting that the infection-associated strains were opportunistic, rather than pathogenic, in nature.

Bioinformatic searches of the *S. salivarius* M18 annotated gene sequence were conducted to identify potentially undesirable phenotypic properties of the organism. Virulence searches using PATRIC online software did not identify any genes encoding known Streptococcal toxins (*e.g*., streptolysin O and S) or antibiotic resistance determinants. Additional bioinformatics analyses of the *S. salivarius* M18 gene assembly for pathogenicity (PathogenFinder 1.1) and antibiotic resistance (ResFinder 3.0) were conducted and the strain was identified as non-pathogenic and no positive alignments to potential antimicrobial resistance genes were identified. Antimicrobial resistance also was tested using ResFinderFG 1.0, resulting in 1 match in the *S. salivarius* M18 whole genome sequence to an uncultured clone (Genbank KF629717.1) with putative resistance to trimethoprim. A Basic Local Alignment Search Tool (BLAST) search of the cloned fragment identified hits with >90% identity to a majority of the *S. salivarius* genomes that have been sequenced, indicating that the genotype is unlikely to represent an acquired resistance trait. In addition to these bioinformatic analyses, *in vitro* antibiotic resistance testing was conducted for *S. salivarius* M18 against a broad range of clinically important antibiotics. Using microdilution and disk diffusion methods, *S. salivarius* M18 was reported to be sensitive to penicillin, ampicillin, amoxicillin, vancomycin, erythromycin, tetracycline, ofloxacin, clindamycin, ceftriaxone, and chloramphenicol, based on guidelines set by the Clinical and Laboratory Standards Institute.

Streptococci are a predominant species in the human oral cavity (Tagg, 2004). The production of bacteriocins by naturally occurring oral streptococci is widespread and ubiquitous among Gram-positive bacteria (Burton *et al.,* 2013a). *S. salivarius* M18 produces 4 bacteriocins, the subtype AII lantibiotics salivaricins A2, 9, MPS, and M (Heng *et al.,* 2011). Genetic determinants located on the megaplasmid appear to be responsible for almost all the bacteriocin-producing capacity of *S*. *salivarius* (Burton *et al.,*2013b), and although transfer of the megaplasmid to other strains is possible, it is expected to occur naturally as bacteriocin production is a common phenotype. Most bacteriocins, including the subtype A

lantibiotics (*e.g.,* salivaricin, nisin), operate *via* surfactant effects, disrupting cell membranes *via* pore formation, which in turn leads to dissipation of proton motive forces, adenosine triphosphate (ATP) depletion, and leakage of intracellular contents (Cleveland *et al.*, 2001). This mechanism of action is distinct from that of pharmacological antibiotics, and therefore, it is not expected that the presence of lantibiotics in the food supply would result in the development or propagation of antibiotic resistance against clinically-important antibiotics.

The Expert Panel noted that *S. salivarius* is a dominant species within the oral microflora and is present in all individuals from birth and throughout life. In humans (and likely most mammals), direct exposure of *S. salivarius* to the systemic circulation through minor and major trauma to the oral mucosa probably occurs on a routine basis in all individuals, across all age groups and population types, including immunocompromised persons. Ubiquitous transfer of *S. salivarius* isolates between individuals through normal social interactions is without adverse effects (Kort *et al*., 2014). Based on its critical evaluation of available information including the aforementioned, the Expert Panel concluded that *S. salivarius* M18 is non-pathogenic, and that the risk of opportunistic infection from the proposed use of *S. salivarius* M18 in food would be no greater than that currently posed by GRAS strains of *Lactobacillus* and *Bifidobacteria* that are currently available in foods on the U.S. market.

Application of the Decision Tree Approach (Pariza *et al.,* 2015)

The Expert Panel unanimously agreed that the decision tree approach to determine the safety of microbial cultures intended for human and animal consumption (Pariza *et al.,* 2015) should be applied to evaluate the safety of *S. salivarius* M18 for use as a food ingredient. Based on the data and information provided in the dossier titled "*Documentation Supporting the Generally Recognized as Safe (GRAS) Status of Streptococcus salivarius M18 for Use as an Ingredient in Conventional Food Products*", utilization of the decision tree resulted in the following conclusion regarding *S. salivarius* M18: "*The strain is deemed safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption*". See Attachment B for the decision tree assessment.

CONCLUSION

We, the undersigned independent qualified members of the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to the safety of the proposed use of *Streptococcus sa/ivarius* (5. *salivarius)* M18 freeze-dried powder as a food ingredient in conventional food and beverage products across multiple food categories (baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogues; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices; soft candy; sweet sauces, toppings, and syrups; and medical foods) at a level of 20 mg per serving (providing a minimum of $1x10^9$ CFU/serving). We unanimously conclude that the proposed use of 5. *sa/ivarius* M18, meeting appropriate food grade specifications and produced in accordance with current Good Manufacturing Practice (cGMP), is safe and suitable and Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

[redacted]

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07 May 2018
Date

[redacted]

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REFERENCES

Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM (2007). Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2(3):285-295 [abstract only].

Booijink CC, El-Aidy S, Rajilić-Stojanović M, Heilig HG, Troost FJ, Smidt H, et al. (2010). High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 12(12):3213-3227. DOI:10.1111/j.1462-2920.2010.02294.x.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, et al. (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 36(6):775-780.

Burton JP, Cowley S, Simon RR, McKinney J, Wescombe PA, Tagg JR (2011). Evaluation of safety and human tolerance of the oral probiotic Streptococcus salivarius K12: a randomized, placebo-controlled, double-blind study. Food Chem Toxicol 49(9):2356-2364.

Burton JP, Wescombe PA, Macklaim JM, Chai MH, Macdonald K, Hale JD, et al. (2013a). Persistence of the oral probiotic *Streptococcus salivarius* M18 is dose dependent and megaplasmid transfer can augment their bacteriocin production and adhesion characteristics. PLoS One 8(6):e65991 [10pp]. DOI:10.1371/journal.pone.0065991.

Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JD, et al. (2013b). Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. J Med Microbiol 62(6):875-884. DOI:10.1099/jmm.0.056663-0.

CDC (2006). *Analytical and Reporting Guidelines: The National Health and Nutrition Examination Survey (NHANES*) . Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/nhanes_analytic_guidelines_dec_2005.pdf.

CDC (2009). *National Health and Nutrition Examination Survey (NHANES): 2003-2004; 2005-2006* . Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2003 ; https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2005.

Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001). Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 71(1):1-20.

Dalidowitz C (2005). Fortified breast milk safety. J Am Diet Assoc 105(10):1572-1573.

Delorme C, Poyart C, Ehrlich SD, Renault P (2007). Extent of horizontal gene transfer in evolution of Streptococci of the salivarius group. J Bacteriol 189(4):1330-1341.

Di Pierro F, Zanvit A, Nobili P, Risso P, Fornaini C (2015). Cariogram outcome after 90 days of oral treatment with *Streptococcus salivarius* M18 in children at high risk for dental caries: results of a randomized, controlled study. Clin Cosmet Investig Dent 7:107-13. DOI:10.2147/CCIDE.S93066.

EFSA (2008). A Report from the Data Collection and Exposure Unit in Response to a Request from the European Commission. Consumption of Food and Beverages with Added Plant Sterols in the European Union. (EFSA/DATEX 03). EFSA J 133r:1-21. Available at: https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2008.133r.

Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002). Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68(1):219-226.

Gibbons RJ, van Houte J (1975). Bacterial adherence in oral microbial ecology. Annu Rev Microbiol 29:19-44.

Heikkilä MP, Saris PE (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. J Appl Microbiol 95(3):471-478.

Heng NC, Haji-Ishak NS, Kalyan A, Wong AY, Lovric M, Bridson JM, et al. (2011). Genome sequence of the bacteriocin-producing oral probiotic *Streptococcus salivarius* strain M18. J Bacteriol 193(22):6402-6403. DOI:10.1128/JB.06001-11.

Kort R, Caspers M, van de Graaf A, van Egmond W, Keijser B, Roeselers G (2014). Shaping the oral microbiota through intimate kissing. Microbiome 17(2):41 [8pp]. DOI:10.1186/2049-2618-2-41.

Leser TD, Mølbak L (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. Environ Microbiol 11(9):2194-2206. DOI:10.1111/j.1462-2920.2009.01941.x.

Martín R, Langa L, Reviriego C, Jiménez E, Marín ML, Olivares M, et al. (2004). Commensal microflora of human milk new perspectives for food bacteriotherapy. Trends Food Sci Technol 15(3&4):121-127.

Martín R, Heilig HG, Zoetendal EG, Jiménez E, Fernández L, Smidt H, et al. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. Res Microbiol 158(1):31-37.

Pariza MW, Gillies KO, Kraak-Ripple SF, Leyer G, Smith AB (2015). Determining the safety of microbial cultures for consumption by humans and animals. Regul Toxicol Pharmacol 73(1):164-171. DOI:10.1016/j.yrtph.2015.07.003.

Park H-K, Shim S-S, Kim S-Y, Park J-H, Park S-E, Kim H-J, et al. (2005). Molecular analysis of colonized bacteria in a human newborn infant gut. J Microbiol 43(4):345-353.

Scariya L, Nagarathna DV, Varghese M (2015). Probiotics in periodontal therapy. Int J Pharm Bio Sci 6(1):P242-P250.

Sundin OH, Mendoza-Ladd A, Zeng M, Diaz-Arévalo D, Morales E, Fagan BM, et al. (2017). The human jejunum has an endogenous microbiota that differs from those in the oral cavity and colon. BMC Microbiol 17(1):160 [17pp]. DOI:10.1186/s12866-017-1059-6.

Tagg JR (2004). Prevention of streptococcal pharyngitis by anti-*Streptococcus pyogenes* bacteriocin-like inhibitory substances (BLIS) produced by *Streptococcus salivarius*. Indian J Med Res 119(Suppl.):13-16.

U.S. FDA (2004). *Food Allergen Labeling and Consumer Protection Act of 2004 (Public Law 108-282, Title II)* [FALCPA]. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106187.htm [Page Last Updated: 05/02/2016].

U.S. FDA (2012). Part 101—Food labeling. §101.12—Reference amounts customarily consumed per eating occasion. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs*. (U.S. Food and Drug Administration). Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR.

U.S. FDA (2016). *Agency Response Letter GRAS Notice No. GRN 000591 [Streptococcus salivarius K12, Dunedin, New Zealand: BLIS Technologies Ltd.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=591 [Jan. 25, 2016].

U.S. FDA (2017). Part 170—Secondary direct food additives permitted in food for human consumption. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR.

USDA (2009). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2003-2004; 2005-2006* . Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release.

Wang M, Ahrné S, Jeppsson B, Molin G (2005). Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol Ecol 54(2):219-231.

WHO/FAO (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Rome, Italy: World Health Organization (WHO) / Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/3/a-a1554e.pdf.

Wilson M, Martin R, Walk ST, Young C, Grossman S, McKean EL, et al. (2012). Clinical and laboratory features of *Streptococcus salivarius* meningitis: a case report and literature review. Clin Med Res 10(1):15-25. DOI:10.3121/cmr.2011.1001.

Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, et al. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J 6(7):1415-1426. DOI:10.1038/ismej.2011.212.

Attachment A
Proposed Food-Uses and Use-Levels for Freeze-Dried *Streptococcus salivarius* M18 Powder in the U.S.

Table A-1 Food-Uses and Use-Levels for *Streptococcus salivarius* M18

Food Category	Proposed Food-Uses	*S. salivarius* M18 Use-Level		Serving Size (g or mL)*	Use-Level (%)
		CFU/serving	mg/serving		
Baby and Toddler Foods	Cereals, Baby Food	$1.0x10^9$	20	15 (dry, instant)[a] 110 (RTS)[a]	0.13 (dry, instant) 0.018 (RTS)
	Cookies, Crackers, and Puffs, Baby/Toddler Food	$1.0x10^9$	20	7[a]	0.10
	RTS Fruit-Based Baby/Toddler Food	$1.0x10^9$	20	60 (strained)[a] 110 (junior)[a] 125 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.016 (toddler)
	Fruit Juices, Baby Food	$1.0x10^9$	20	125[a]	0.016
	RTS Dinners, Baby/Toddler Food	$1.0x10^9$	20	60 (strained)[a] 110 (junior)[a] 170 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.012 (toddler)
	RTS Desserts, Baby Food	$1.0x10^9$	20	60 (strained) 110 (junior)	0.03 (strained) 0.018 (junior)
	RTF Vegetable-Based Baby/Toddler Food	$1.0x10^9$	20	60 (strained) 110 (junior) 70 (toddler)	0.03 (strained) 0.018 (junior) 0.029 (toddler)
Baked Goods and Baking Mixes	Cookies (chocolate coating)	$1.0x10^9$	20	20	0.10
Beverages and Beverage Bases	Meal Replacement powders (fortified, protein, and mineral replenish)	$1.0x10^9$	20	16 to 40	0.05 to 0.13
	Sports and Energy Drinks	$1.0x10^9$	20	250	0.01
	Water (Still or Mineral)	$1.0x10^9$	20	237	0.01
Breakfast Cereals	Breakfast Cereals	$1.0x10^9$	20	29	0.07
	Muesli and Dry Blended Cereals	$1.0x10^9$	20	85	0.02
Cheeses	Natural Cheeses	$1.0x10^9$	20	20 to 30	0.07 to 0.10
Chewing Gum	Chewing Gum	$1.0x10^9$	20	3	0.67
Dairy Product Analogues	Milk Substitutes	$1.0x10^9$	20	244	0.01
Frozen Dairy Desserts and Mixes	Frozen Yogurt	$1.0x10^9$	20	174	0.02
	Ice Cream	$1.0x10^9$	20	66	0.03
Gelatins, Puddings, and Fillings	Custards (pourable)	$1.0x10^9$	20	113	0.02
	Dessert Mixes (powder)	$1.0x10^9$	20	25	0.08
Grain Products and Pastas	Granola and Breakfast Bars	$1.0x10^9$	20	28	0.07
	Protein Bars	$1.0x10^9$	20	68	0.03
Hard Candy	Mint Candies	$1.0x10^9$	20	25	0.08
Milk, Whole and Skim	Milk (flavored, pasteurized)	$1.0x10^9$	20	244	0.01
	Milk (fresh)	$1.0x10^9$	20	244	0.01
	Milk Powder (skim or whole)	$1.0x10^9$	20	23 to 32	0.06 to 0.09
Milk Products	Cream (pasteurized)	$1.0x10^9$	20	244	0.01
	Cultured Milk Products	$1.0x10^9$	20	180	0.01
	Dairy Desserts	$1.0x10^9$	20	100 to 180	0.01 to 0.02
	Milkshake Mixes (powder)	$1.0x10^9$	20	21	0.10
	Yogurt	$1.0x10^9$	20	227	0.01
	Yogurt Drinks	$1.0x10^9$	20	244	0.01

Table A-1 Food-Uses and Use-Levels for *Streptococcus salivarius* M18

Food Category	Proposed Food-Uses	*S. salivarius* M18 Use-Level		Serving Size (g or mL)*	Use-Level (%)
		CFU/serving	mg/serving		
Nuts and Nut Products	Peanut Butter	$1.0x10^9$	20	32	0.06
Processed Fruits and Fruit Juices	Fruit-Flavored Beverages (powder)	$1.0x10^9$	20	18	0.11
	Fruit Juices	$1.0x10^9$	20	263	0.01
	Fruit Juice Drinks	$1.0x10^9$	20	209	0.01
Soft Candy	Chewable Lozenges	$1.0x10^9$	20	3	0.67
	Chocolate Bars	$1.0x10^9$	20	44	0.05
	Soft Gel and Rapid Melt Technologies	$1.0x10^9$	20	2	1
Sweet Sauces, Toppings, and Syrups	Cinnamon, Nutmeg, and Chocolate Sprinkle	$1.0x10^9$	20	4[a]	0.50
	Sugar and Sweetener Sprinkle	$0.5x10^9$	10	4[a]	0.25

CFU = colony-forming units; RTF = ready to feed; RTS = ready to serve.
*Serving sizes were provided by BLIS Technologies, unless otherwise indicated.
[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the United States Code of Federal Regulations (21 CFR §101.12 – U.S. FDA, 2012).

Attachment B
Decision Tree Assessment

An evaluation of the safety of *S. salivarius* M18 for human consumption was conducted using the Decision Tree approach described by Pariza and colleagues (Pariza *et al*., 2015).

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).

YES. The whole genome and plasmid has been sequenced and is deposited in a publicly available database. *S. salivarius* M18 also has been subject to classical phenotypic and genotypic characterization.

2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.

YES.

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)

YES. Bioinformatic analyses of the whole genome have demonstrated that the strain is free of classical *Streptococcus* virulence factors and is absent of genes potentially conferring pathogenic traits to the organism.

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)

YES. The absence of functional and transferable antibiotic resistance genes has been demonstrated using bioinformatic analyses and classical *in vitro* analyses using the E-test and agar dilution assays.

5. Does the strain produce antimicrobial substances? (If NO, go to 6. If YES, go to 15.)

NO. The strain is known to produce bacteriocins (*e.g*., salivaricins) that are antagonistic to *Streptococcus* sp.; however, there is no documented evidence of the species *S. salivarius* producing antibiotics with structure activity behaviors that would be of clinical importance.

6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a or 7b. If NO, go to 8a

NO.

8a. For strains to be used in human food was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? (If YES, go to 9a. If NO, go to 13a.)

NO; however, the strain is a human commensal obtained from saliva sample of healthy volunteer therefore it is considered appropriate to proceed to 9a.

9a. For strains to be used in human food: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts? (If YES, go to 10a. If NO, go to 13a.)

YES. *S. salivarius* K12 has GRAS status as described in GRN 591 (U.S. FDA, 2016).

10a. For strains to be used in human food: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food? (If YES, go to 11a. If NO, go to 13a.)

YES.

11a. For strains to be used in human food: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12a. If YES, go to 13a.)

NO. Ingestion of *S. salivarius* strains is ubiquitous in the general population, including at risk individuals, through consumption of saliva (*i.e., S. salivarius* is typically present at levels of around 1×10^7 CFU/mL of saliva). Transfer of *S. salivarius* strains between humans through normal social interactions also is not associated with safety concerns.

12a. For strains to be used in human food: Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14a. If YES, go to 13a.)

No. Food-uses are consistent with those described under GRN 591 (U.S. FDA, 2016) for *S. salivarius* K12 and the expanded intake of the species is not expected.

13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? If YES, go to 15. If NO, go to 14a.)

No. There are no anticipated physiological effects of consuming the strain outside of potential transient changes in the oral microflora composition. These strain specific effects of *S. salivarius* M18 have been evaluated in human colonization studies and no adverse changes in the oral flora composition or other adverse physiological effects have been reported in the studies.

14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

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Global Experts in Food Assurance

Certificate of Analysis

Final Report

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Report Issued: 23-Nov-2018 **AsureQuality Reference: 18-293003** **Sample(s) Received:** 23-Nov-2018 08:45

Results

The tests were performed on the samples as received.

Customer Sample Name: M1845 **AsureQuality ID:** 18-293003-1

Batch/Lot No.: M1845 **Sample Condition**: Acceptable

Test	Result	Unit	Method Reference
Beta-Lacto Globulin	>1.00	ppm	ELISA Systems
Milk Casein	3.4	ppm	ELISA Systems

Analysis Summary

Auckland Laboratory

Analysis	Method	Accreditation	Authorised by
Beta lactoglobulin Allergen			
NT-BLG-01, 01-DEFAULT	ELISA Systems	IANZ	Tim Hudson
Milk Casein Allergen			
NT-MILK01, 01-DEFAULT	ELISA Systems	IANZ	Tim Hudson

Tim Hudson
Laboratory Analyst

Accreditation

IANZ
ACCREDITED LABORATORY

