ORIGINAL SUBMISSION

BLIS Technologies

10 Birch Street
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July 02, 2015

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:
Re: GRAS Exemption Claim for *Streptococcus salivarius* K12

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [BLIS Technologies Ltd., 10 Birch St, PO Box 5804, Dunedin, New Zealand], a notice of the determination, on the basis of scientific procedures, that *Streptococcus salivarius* K12, as defined in the enclosed documentation, is GRAS under specific conditions of use as a food ingredient in specified food and beverage products, and therefore, are exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of *Streptococcus salivarius* K12 under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Exemption Claim for *Streptococcus salivarius* K12" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

Should you have any questions or concerns regarding this GRAS Notification, 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,

(b) (6)

Barry Richardson

CEO

GRAS Exemption Claim for *Streptococcus salivarius* K12

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

BLIS Technologies Ltd.
10 Birch St,
PO Box 5804
Dunedin
New Zealand

April 06, 2015

GRAS Exemption Claim for *Streptococcus salivarius* K12

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I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR § 170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

Streptococcus salivarius K12 has been determined by BLIS Technologies Ltd. (BLIS hereafter) to be Generally Recognized as Safe (GRAS) under the conditions of intended use in food as described in Section I.D., consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food. Therefore, the use of *Streptococcus salivarius* K12 in food as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Barry Richardson
BLIS Technologies Ltd.

28 May 2015.
Date

I.B Name and Address of Notifier

BLIS Technologies Ltd.
10 Birch St,
PO Box 5804
Dunedin
New Zealand

I.C Common Name of the Notified Substance

Streptococcus salivarius K12

I.D Conditions of Intended Use in Food

BLIS intends to market freeze-dried powder of *Streptococcus salivarius* (*S. salivarius*) K12 powder as a food ingredient in the United States (U.S.) for use in conventional food and beverage products across multiple food categories including baby, infant[1], and toddler foods; baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses;

[1] Infant foods excluding infant formula

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; and medical foods) at levels of up to 20 mg/serving (providing a minimum of $2x10^9$ colony forming units (CFU)/serving).

The individual proposed food uses and use levels for freeze dried *S. salivarius* K12 are summarized in Table I.D-1 below.

Table I.D-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Streptococcus salivarius* K12 in the United States (NHANES 2003-2006)

Food Category	Proposed Food-Uses	*S. salivarius* K12 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 Analogs	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

Table I.D-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Streptococcus salivarius* K12 in the United States (NHANES 2003-2006)

Food Category	Proposed Food-Uses	*S. salivarius* K12 Use-Level		Serving Size (g or mL)*	Use-Level (%)
		CFU/serving	mg/serving		
Gelatins, Puddings, and Fillings	Custards (pourable)[b]	$1.0X10^9$	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
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[c]	$1.0X10^9$	20	3	0.67
	Soft Gel and Rapid Melt Technologies[d]	$1.0X10^9$	20	2	1
	Sugar and Sweetener Sprinkle	$0.5x10^8$	10	4[a]	0.25

RTD = ready to drink; RTE = ready to eat; 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 U.S. CFR (21 CFR § 101.12 – U.S. FDA, 2014).
[b] No food codes for custard (pourable) is available in NHANES 2003-2006; however, surrogate codes (custard-filled products) that have a similar composition and pattern of use will be used to represent this category.
[c] No food codes for chewable lozenges is available in NHANES 2003-2006; however, the exposure to chewable lozenges is expected to be similar to the exposure from mint candies, which is already included as one of the proposed food-uses.
[d] No food codes for soft gel and rapid melt technologies were identified in NHANES 2003-2006.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2014), the proposed uses of *S. salivarius* K12 as an ingredient has been determined by BLIS to be GRAS on the basis of scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

BLIS Technologies Ltd.
10 Birch St,
PO Box 5804
Dunedin
New Zealand

Attention:
Barry Richardson Ph.D.
CEO
BLIS Technologies Ltd.

Barry.richardson@blis.co.nz

Should the FDA have any questions or additional information requests regarding this notification, BLIS will supply these data and information.

II. DETAILED INFORMATION ON IDENTITY AND MANUFACTURING OF *STREPTOCOCCUS SALIVARIUS* K12

II.A Common Name and Taxonomic Lineage

Common Name: *Streptococcus salivarius* K12

Trade Name: BLIS K12

Taxonomic Lineage:

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Lactobacillales
Family: Streptococcaceae
Genus: *Streptococcus*
Species: *salivarius*
Strain: K12

II.B History of *Streptococcus salivarius* K12

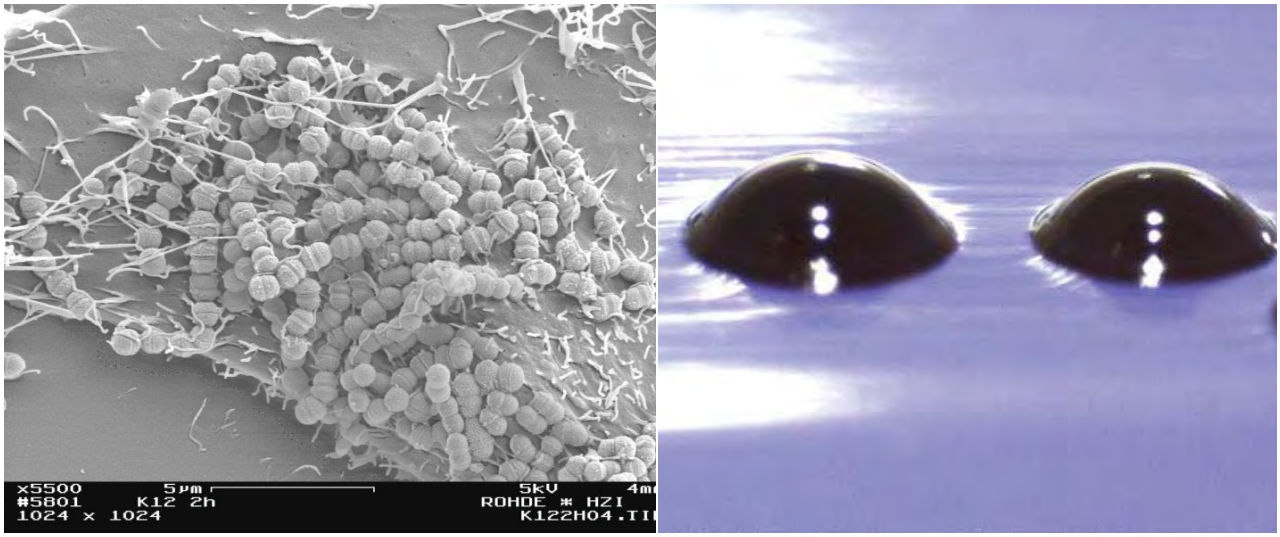
S. salivarius K12 was isolated from a saliva sample taken from a healthy Dunedin school child in 1989. *S. salivarius* K12 has been deposited in the American Type Culture Collection (ATCC) as ATCC BAA 1024. It also has been lodged with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkuturen GmbH) under the accession number DSM 13084. The species and strain identity have been characterized using the most current phenotypic and genotypic techniques, as discussed in Sections II.C and II.D below.

II.C Phenotypic Characterization

Streptococci are spherical, non-motile, Gram-positive microorganisms that occur in chains or pairs (Figure II.C-1). Typically the identification of *S. salivarius* K12 from a sample (heterogeneous bacteriological sample) starts with selection of a semi-selective bacteriological agar (Mitis-Salivarius Agar). Specific *S. salivarius* distinctive colony types on the agar are then isolated and re-propagated on non-specific bacteriological media for further identification procedures. These procedures include conventional biochemical techniques such as the API20 and API50CH tests described below.

Figure II.C-1 Colony Morphology of *Streptococcus salivarius* sp.

A **B**



A) Electron micrograph of *S. salivarius* K12. **B)** Growth of *S. salivarius* sp. on Mitis-Salivarius agar (right) results in raised blue colonies.

1. Carbohydrate Fermentation

The sugar fermentation profile of *S. salivarius* K12 was determined using the API 50 CH test system (bioMérieux), and is presented in Table II.C-1. The test system, which analyzes 49 different sugars to determine carbohydrate metabolism, has been validated for variability introduced by inoculum, age, and cell density.

Table II.C-1 API 50 Carbohydrate Fermentation Profile of *Streptococcus salivarius* K12

Substrate	Result	Substrate	Result
Glycerol	-	Salicin	+
Erythritol	-	D-cellobiose	+
D-arabinose	-	D-maltose	+
L-arabinose	-	D-lactose	+
D-ribose	-	D-melibiose	-
D-xylose	-	D-saccharose	+
L-xylose	-	D-trehalose	+
D-adonitol	-	Inulin	+
Methyl-β-D-xylopranoside	-	D-melezitose	-
D-galactose	+	D-raffinose	+
D-glucose	+	Amidon	-
D-fructose	+	Glycogen	-
D-mannose	+	Xylitol	-
L-sorbose	-	Gentiobiose	-
L-rhamnose	-	D-turanose	-

Table II.C-1 API 50 Carbohydrate Fermentation Profile of *Streptococcus salivarius* K12			
Substrate	**Result**	**Substrate**	**Result**
Dulctiol	-	D-Xylose	-
Inositol	-	D-Tagatose	+
D-Mannitol	-	D-Fucose	-
D-Sorbitol	-	L-Fucose	-
Methyl-α-D-mannopyranoside	-	D-Arabitol	-
Methyl-α-D-glucopyranoside	-	L-Arabitol	-
N-Acetylglucosamine	+	Gluconate	-
Amygdalin	-	2-Ketogluconate	-
Arbutine	+	5-Ketogluconate	-
Esculin	-		

2. Enzyme Profile

The enzyme profile of *S. salivarius* K12 was determined using the API 20 Strep test system (bioMérieux), which includes 20 wells containing the following biochemical tests in dehydrated form, including 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 II.C-2, *S. salivarius* K12 tested positive for the following enzymes: acetoin production, β-glucosidase, alkaline phosphatase, leucine aminopeptidase, D-lactose, D-trehalose, inulin, and D-raffinose.

Table II.C-2 API 20 Strep Enzyme Profile for *Streptococcus salivarius* K12	
Enzyme Reaction	***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	-

Table II.C-2 API 20 Strep Enzyme Profile for *Streptococcus salivarius* K12	
Enzyme Reaction	***S. salivarius* K12**
D-Lactose	+
D-Trehalose	+
Inulin	+
D-Raffinose	+
Starch	-
Glycogen	-
β-Hemolysis	-

II.D Genotypic Characterization

1. Genome Sequencing and Species Characterization

A high quality draft genome (chromosomal and megaplasmid DNA) of *S. salivarius* K12 was determined using 454 GS-FLX sequences (Roche Diagnostics) and Solexa HiSeq (Illumina, Inc.) (Barretto *et al*., 2012). Chromosome sequencing produced 95 contigs with depth of coverage of 44x. Contigs were aligned with the genome of *S. salivarius* JIM8777 and gaps closed using PCR followed by Sanger sequencing and primer walking. Using the gene sequence data, the nucleotide sequence for the complete 16S rRNA gene for *S. salivarius* K12 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 *Streptococcus thermophilus*. Specifically, comparison of the 1,537 base pairs of the *S. salivarius* K12 sequence indicated there were little differences compared to *S. salivarius* (99.8% homologous) and *S. thermophilus* (99.61% homologous).

Annotation of the gene sequence was conducted by IG Assets. Two thousand and eighty-nine (2,089) protein coding sequences were identified on the chromosome with 82% annotated to proteins with known biological function, and 18% to hypothetical proteins. The megaplasmid was determined to contain 69 genes (42%) with known biological function and 95 (68%) encoded for hypothetical proteins (Barretto *et al*., 2012).

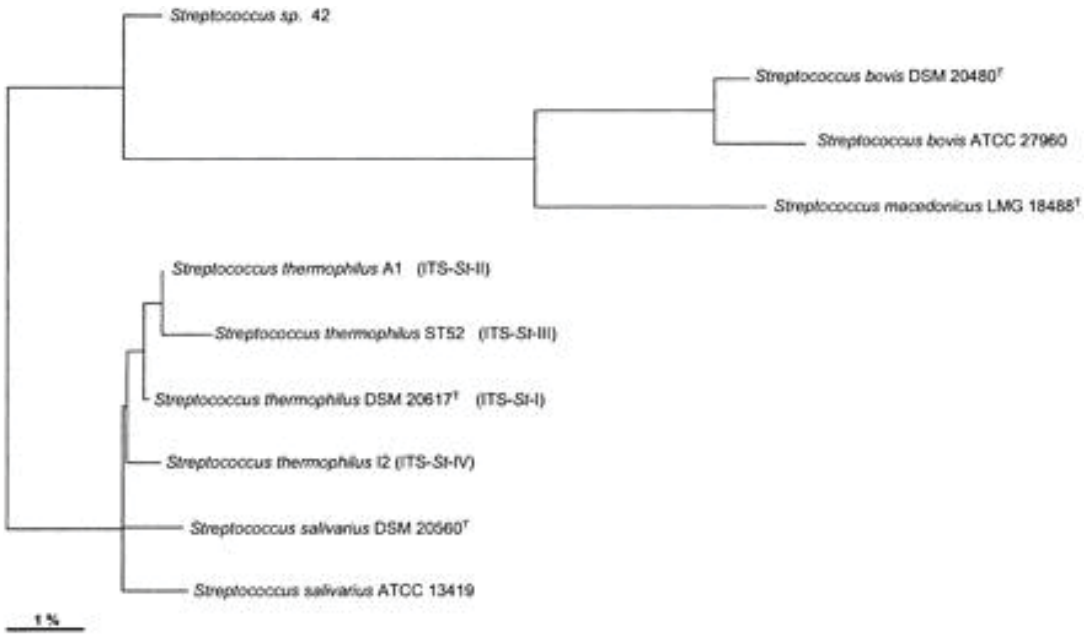
2. Genetic Similarity to the Yogurt Starter *Streptococcus thermophilus*

The species *Streptococcus thermophilus* was first described and named by Orla-Jensen in 1919 during studies of the bacteriology of milk and dairy products; however, the taxonomy of the species and genetic relatedness to *S. salivarius* has been a matter of controversy for many years (Farrow and Collins, 1984). In 1984 Farrow and Collins presented studies questioning the naming of S. thermophilus as distinct species (Farrow and Collins, 1984). Using DNA base composition, DNA-DNA homology, and long-chain fatty acid analyses, the authors demonstrated that *S. thermophilus* and *S. salivarius* possess similar mol % G + C values (about

37 to 41) and long-chain fatty acid profiles, and belonged to a single DNA homology group. On the basis of this information and earlier studies, the authors proposed that *S. thermophilus* be reclassified as *S. salivarius* subsp. *thermophilus* comb. nov. (Farrow and Collins, 1984; Moore *et al*., 1985). Several years later findings by Schleifer *et al*. (1991) using DNA-DNA re-association experiments under more stringent conditions demonstrated that *S. thermophilus* was most likely a distinct species. Based on their findings, Schleifer *et al.* (1991) proposed that the taxonomic name of the species should be shifted back to the former name *Streptococcus thermophilus* (Schleifer *et al.*, 1991; IJSB, 1995). However, despite recent findings by Shleifer and colleagues, the Farrow and Collins (1984) taxonomic designation of *S. salivarius* subsp. *thermophilus* is still widely used by various international culture collection bodies that maintain curated taxonomic catalogs such as ATCC Biological Resource Center and Leibniz-Institut DSMZ German Collection of Microorganism and Cell Cultures (ATCC, 2014; DSMZ, 2014). *S. salivarius* subsp. *thermophilus* therefore remains and appropriate basonym for the species.

Additional evidence supporting the close genetic relationship between *S. salivarius* and *S. thermophilus* is the similarity of their 16S rRNA sequences. According to Bentley *et al.* (1991), species of the *Streptococcus* genera can be grouped into 6 divisions based upon their homology of 16S rRNA gene sequences. The group V cluster consists of the following species: *S. salivarius, S. vestibularis,* and *S. thermophilus* (Bentley *et al.*, 1991). Using 16S–23S rRNA intergenic spacer region sequence analysis, it can be shown that *S. thermophilus* and related dairy species are clustered in 2 main branches – one containing *S. macedonicus* and *S. bovis*, and one containing *S. thermophilus* and *S. salivarius*. This relationship is depicted in Figure II.D-1 using neighbor-joining tree analyses of the 16S–23S rRNA ITS sequences.

Figure II.D-1 Neighbor-Joining Tree Based on 16S–23S rRNA ITS Sequences

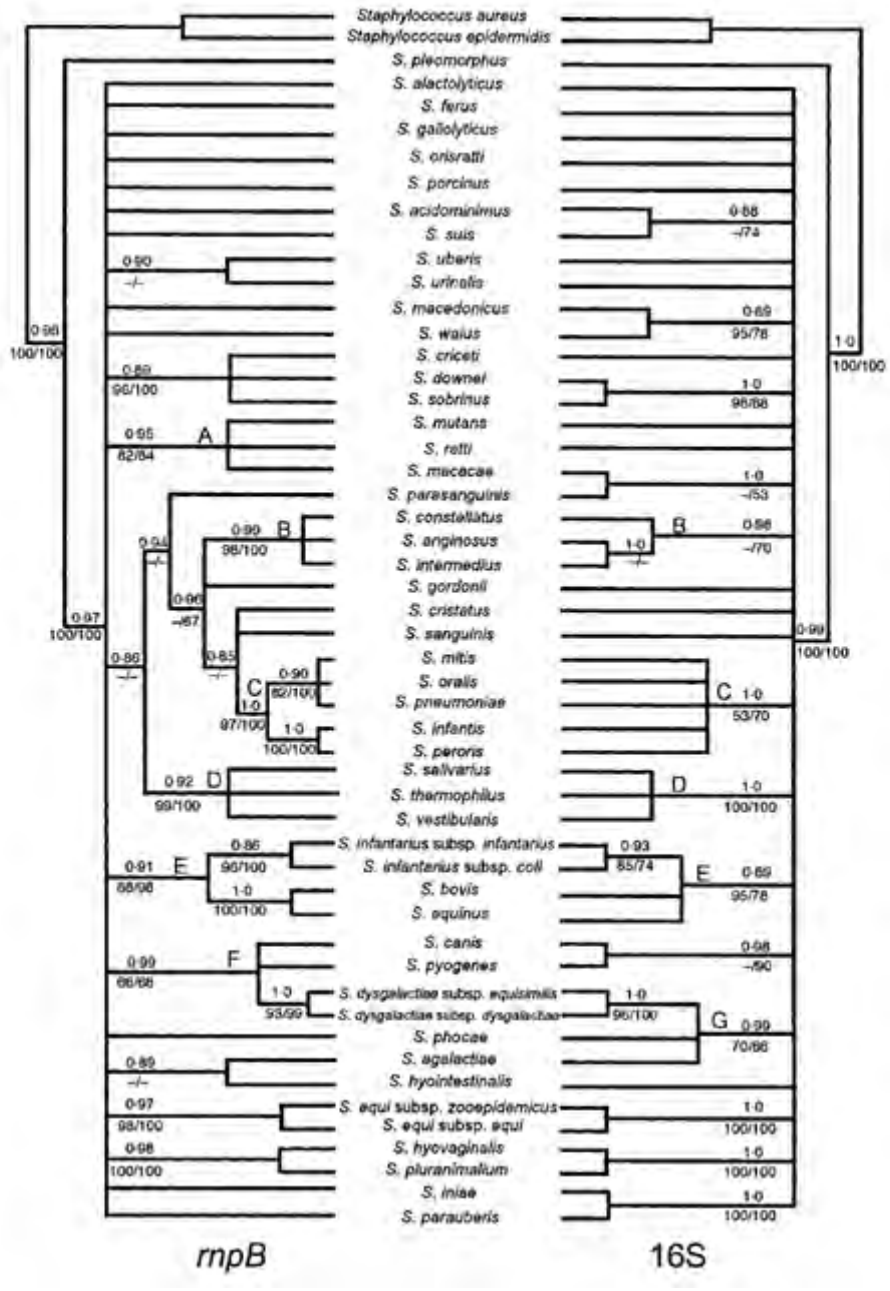


Additional taxonomic evaluations using the *rnpB* gene encoding the RNA subunit of RNase P were reported by Täpp *et al.* (2003). This gene has been shown to be an excellent target for differentiation of bacterial species of the diverse genera, and is suitable for phylogenetic analysis of closely related taxa. Compared to the 16S rRNA gene, rnpB has a higher information value per nucleotide position that, in combination with its short length, gives it potential for species discrimination. Only 7 nucleotides of the 400 bp gene sequence were different between *S. thermophilus* and *S. salivarius* (greater than 98% similarity), and these organisms were shown to be amongst the most highly-related *Streptococci* (Täpp *et al.*, 2003; Innings *et al.*, 2005). These observations are in contrast to the divergent genetic relationship between members of the *S. thermophilus*/*salivarius* group and *streptococcal* pathogens (*e.g., S. pyogenes, S. pneumoniae*). Phylogenetic comparisons of rnpB, 16S rRNA, and both genes combined are shown in Figures II.D-2 and II.D-3 below. Similar studies characterizing the close genetic relationship between *S. salivarius* and *S. thermophilus* and distinct genetic divergence of these species to pathogenic members of the *Streptococci* also have been reported by Pombert *et al*. (2009).

The group V Streptococcus cluster consists of *S. salivarius, S. vestibularis,* and *S. thermophilus.* Extensive phylogenetic analyses of these species not only demonstrate the close genetic similarity between these species but also support the conclusion that these organisms share a common evolutionary lineage: *S. salivarius* and *S. vestibularis* as colonizer's of the human oral mucosa and *S. thermophilus*, the dairy starter culture, originating from direct or indirect invasion of the bovine mammary mucosa by an ancestor of *S. salivarius* or *S. vestibularis* (Pombert *et al.*, 2009). 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 that the evolution of pathogenic traits has not occurred in this lineage.

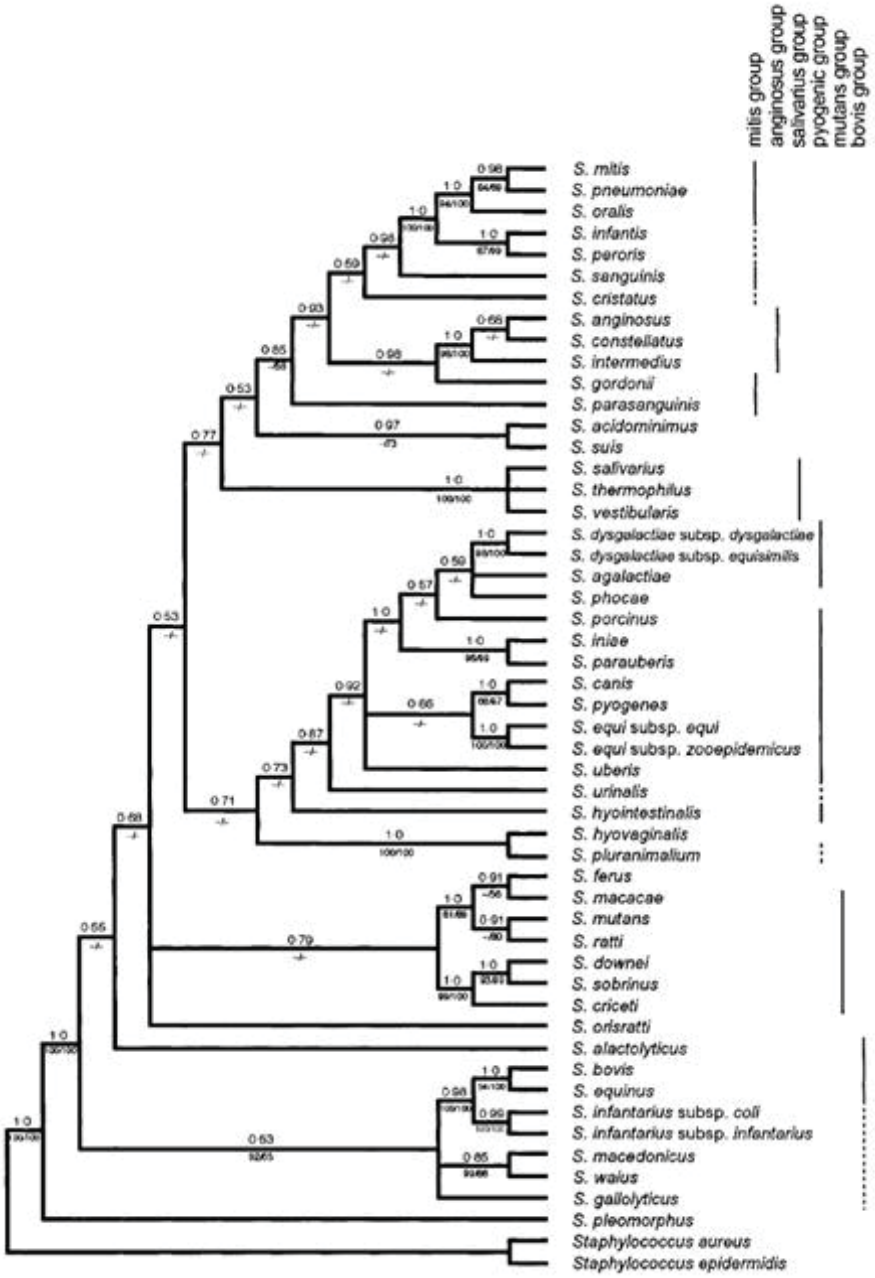
Overall, phylogenetic analyses are sufficiently robust to definitively exclude *S. salivarius* sp. from expressing pathogenic traits characterizing of pathogenic members of the genus.

Figure II.D-2 Phylogenetic Analysis of rnpB and 16S rRNA Genes



Adapted from Täpp *et al.* (2003).

Figure II.D-3 Phylogenetic Analysis of rnpB and 16S rRNA Genes Combined



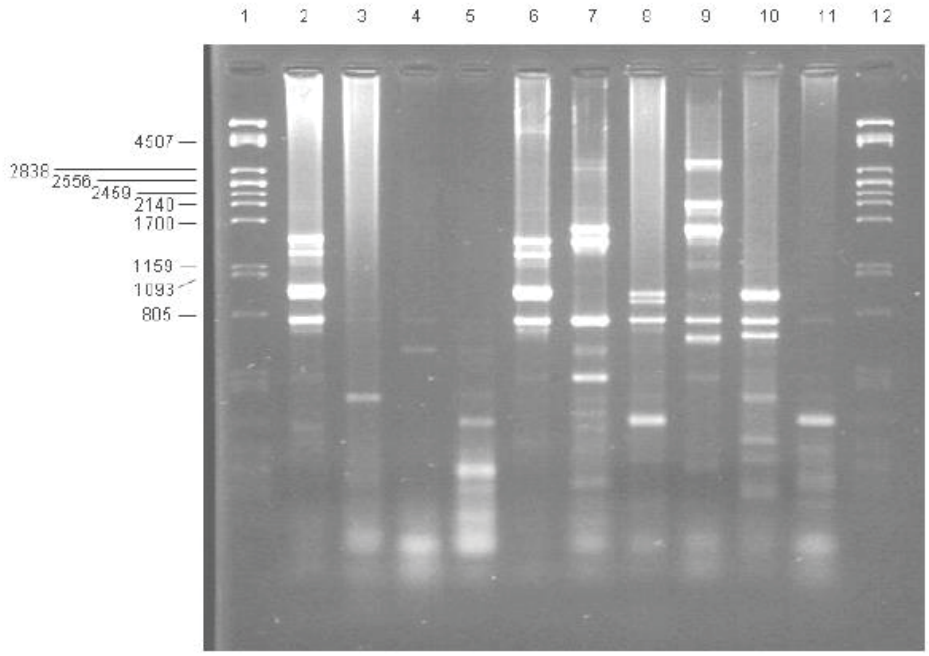
Adapted from Täpp *et al.* (2003).

3. Strain Characterization

3.1 ERIC-PCR Profile of S. salivarius K12

Using Enterobacterial Repetitive Intergenic Consensus (ERIC)-polymerase chain reaction (PCR) molecular typing, a strain-specific genomic fingerprinting method capable of resolving *S. salivarius* K12 at the strain level was developed. As shown in Figure II.D-4, the ERIC-PCR analysis produced a gel electrophoresis fragment pattern that was specific to *S. salivarius* K12.

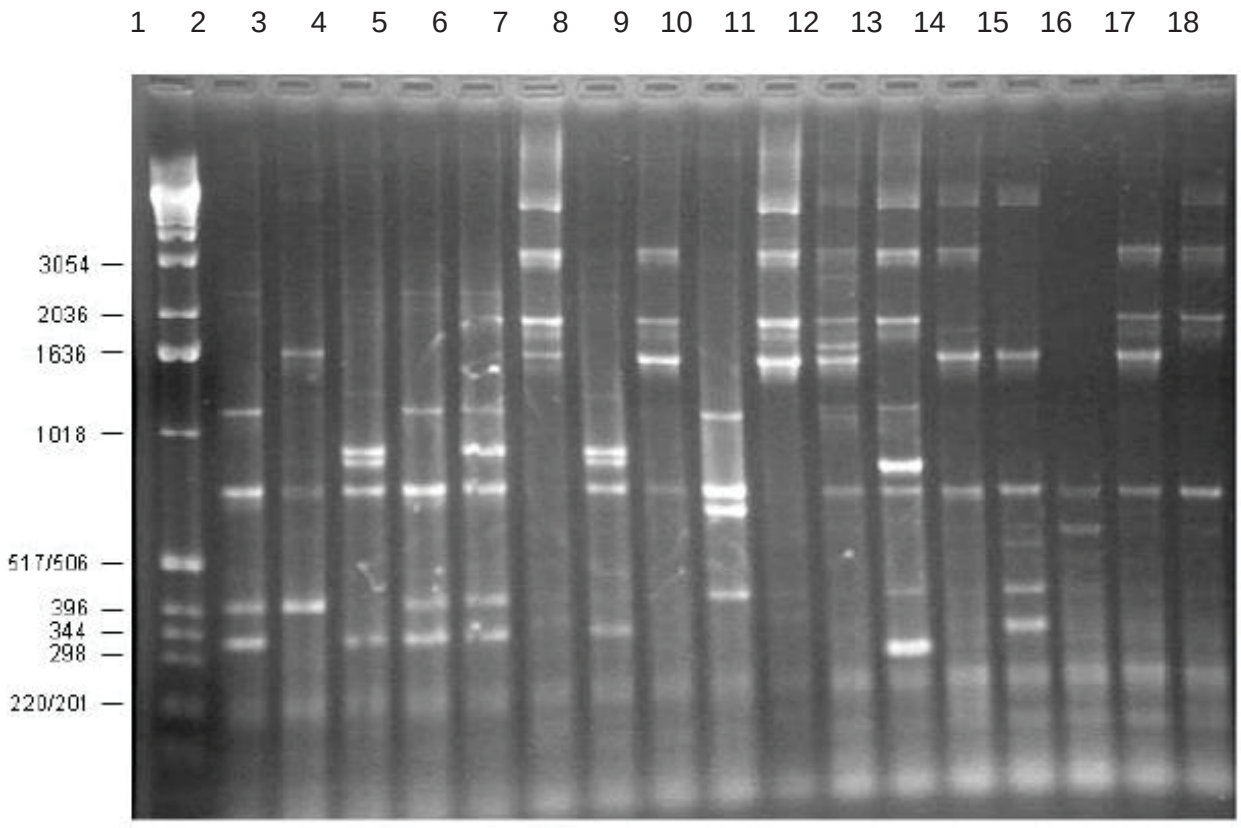
Figure II.D-4 ERIC-PCR Profile of Various *Streptococcus salivarius* Strains



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 (Lane 9), Mia and ToveR, respectively.

Tagg and Bannister (1979) developed a method for P-typing *S. salivarius* isolates, based on their production of bacteriocin-like inhibitory substances. The technique is employed using a set of 9 standard indicator strains susceptible to these substances, and isolates that inhibit all indicator strains are designated as P-type 777 (*i.e.*, *S. salivarius* K12). As indicated in Figure II.D-5, the ERIC-PCR profiles also demonstrate that *S. salivarius* K12 is distinguishable from other P-type 777 *S. salivarius* strains.

Figure II.D-5 ERIC-PCR Profile of 777 P-type *Streptococcus salivarius* Strains

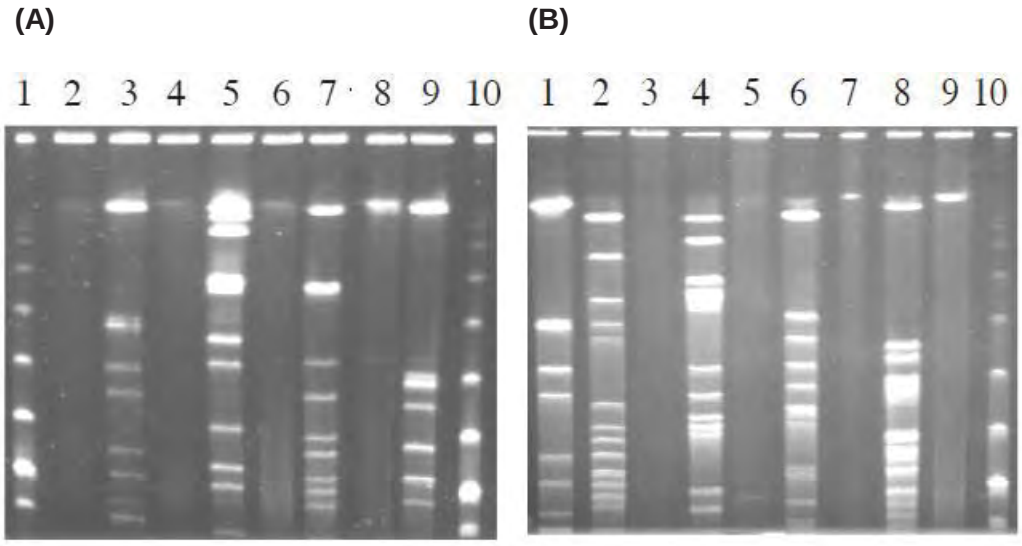


Lane 1, DNA MW marker; Lane 2-18 777 P-type *S. salivarius* strains; Pirie, H21, Min5, SO1, JA, SF1, Strong A, K12 (Lane 9), K104, K28, 1-1, G29, K30, H28, H25, K19, KA, respectively.

3.2 PFGE Profile of S. salivarius K12

Pulsed Field Gel Electrophoresis (PFGE) of restricted chromosomal DNA is the current gold standard for bacterial strain differentiation. The unique SmaI-PFGE profile of *S. salivarius* strain K12 was determined and compared with other *S. salivarius* strains and other streptococcal species (see Figure II.D-6 below). Similar to observations derived using ERIC-PCR, PFGE analyses generated a fingerprint profile that was specific to *S. salivarius* K12.

Figure II.D-6 PFGE Analysis of *Streptococcus salivarius* K12



(A) *Streptococcus salivarius* (lanes 2 to 9) undigested and digested with SmaI in adjacent wells for each strain. Lanes 1 and 10 contain low range PFGE molecular weight marker; (B) Lane 1, *S. salivarius* K12 SmaI digested

II.E Method of Manufacture

1. Raw Materials and Processing-aids

The raw materials and processing aids used in the manufacturing of freeze-dried *S. salivarius* K12 powder are presented in the subheadings below.

1.1 Streptococcus salivarius K12

S. salivarius K12 was isolated from a saliva sample taken from a healthy Dunedin school child in 1989. *S. salivarius* K12 has been lodged with Deutsche Sammlung von Mikroorganismen und Zellkuturen GmbH (accession number DSM 13084), and in American Type Culture Collection (ATCC BAA 1024). Master cultures and working cultures are maintained at BLIS Technologies Ltd., and the Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand. Storage of master and working cultures under controlled conditions also is maintained at Fonterra Cooperative Group Ltd, Microbial Fermentation Unit, Dairy Farm Road, Palmerston North, New Zealand.

1.2 Trehalose

Trehalose is used as a lyoprotectant in the manufacture of the freeze-dried *S. salivarius* K12 powder. Trehalose has been self-affirmed as GRAS for use in foods in general, for multiple

technical effects at levels in accordance with current Good Manufacturing Practice (cGMP) (GRN Notice No. 45) (U.S. FDA, 2000).

1.3 Lactitol

Lactitol is used as a lyoprotectant in the manufacture of the freeze-dried *S. salivarius* K12 powder. Lactitol is a disaccharide polyol (sugar alcohol), derived from lactose. This ingredient has been determined to be GRAS for specified uses as a direct food additive (U.S. FDA, 1995), and foods containing lactitol are eligible for a health claim related to dental caries (*e.g.*, lactitol used to sweeten this food may reduce the risk of dental caries) (21 CFR § 101.80 - U.S. FDA, 2014). The lactitol used in the production of freeze-dried *S. salivarius* K12 powder meets the FCC specifications for lactitol.

1.4 Maltodextrin

Maltodextrin is used as a lyoprotectant in the manufacture of the freeze-dried *S. salivarius* K12 powder. Maltodextrin is direct food substance affirmed as GRAS in foods with no limitation other than cGMP (21 CFR § 184.1444 - U.S. FDA, 2014).

1.5 Ammonia

Ammonia is used as a pH-adjusting agent in the manufacture of the freeze-dried *S. salivarius* K12 powder. Although there is no U.S. regulation permitting the direct use of ammonia itself in foods, ammonia is listed in the “Everything Added to Food in the United States” (EAFUS) database (U.S. FDA, 2011). In addition, several ammonia salts (*i.e.,* ammonium bicarbonate, ammonium chloride, ammonium hydroxide, ammonium citrate, dibasic, ammonium phosphate, monobasic and dibasic) have been affirmed as GRAS by the FDA for use in foods (21 CFR § 184.1135, 184.1138, 184.1139, 184.1140, 184.1141a, 184.1141b – U.S. FDA, 2014).

1.6 Sodium Bicarbonate

Sodium bicarbonate is used as a pH-adjusting agent in the manufacture of the freeze-dried *S. salivarius* K12 powder. The FDA has affirmed sodium bicarbonate to be GRAS in foods with no limitation other than cGMP (21 CFR § 184.1736 – U.S. FDA, 2014)

1.7 Ingredients Used in the Fermentation Medium

The ingredients used in the fermentation medium are listed in Table II.E-1 with confirmation of their regulatory status in food for humans in the U.S.

Table II.E-1 Raw Materials Used in the Fermentation Medium for Production of *Streptococcus salivarius* K12

Raw Material	Purpose	Regulatory Status (U.S. FDA, 2014)
Dextrose Monohydrate	Fermentation Ingredient	21 CFR § 168.111 – Dextrose monohydrate. Standardized sweetener and table syrup.
Yeast Extract (Gistex Fermentation Powder)	Fermentation Ingredient	21 CFR § 184.1983 – Bakers' yeast extract. Used as a flavoring agent and adjuvant at a level not to exceed 5% in food.
Skim milk powder	Fermentation Ingredient	n/a
Polysorbate 80 (Liposorb O-20)	Anti-foaming agent	21 CFR § 172.840 – Polysorbate 80. Food additive permitted for direct addition to a variety of foods.
Neutrase Protease from *Bacillus amyloliquefaciens*	Hydrolysis Enzyme (hydrolyzes proteins present in skim milk powder and yeast extract)	21 CFR § 184.1150 – Bacterial derived protease enzyme preparation. Used as an enzyme in food at levels not to exceed cGMP.
Sodium Hydroxide	pH adjusting agent	21 CFR § 184.1763 – Sodium hydroxide. Used in food with no limitation other than cGMP.

CFR = Code of Federal Regulations; cGMP = current good manufacturing practices; EAFUS = everything added to food in the United States; n/a = not applicable

2. Manufacturing Process

BLIS' *S. salivarius* K12 ingredient is produced using cGMP, using food-grade ingredients and processing aids that have previously been determined to be GRAS, or are used in compliance with appropriate federal regulations. Quality control methods are implemented throughout various stages of fermentation process to ensure production of a pure culture that is devoid of any contaminating pathogens. A brief overview of the manufacture is presented below.

Preparation of Working and Master Cultures

The master and working seed cultures are prepared as follows:

1) An agar slope of *S. salivarius* K12 is received from BLIS.

2) A scraping of growth from the slope is inoculated with 10 mL sterile 10% reconstituted skim milk powder (RSM) and grown overnight at 37°C until coagulation occurs.

3) Sterile 10% RSM is inoculated with 2% of the overnight culture and incubated at 37°C for 6 hours.

4) The culture is then dispensed into 1.5 mL cryovials in laminar flow cabinet. Five vials are held as master stock, 5 vials are held as working stock, 5 vials are held as production unit seed stock, and ~20 vials go to the production unit as working stock for the production of commercial batches. The vials are then stored in a -80°C freezer. No other lyoprotectants are added to the culture as there is sufficient lactose in the RSM.

The following purity checks are performed:

a) Gram stain check for Gram positive cocci

b) Streak onto: Tryptone blood agar (TBA)
Bromocresol purple differential agar (BCP)
de Man, Rogosa Sharpe agar (MRS)
Kelper and McKay agar (KM)
All media incubated at 37°C for 48 hours.
Terzaghi & Sandine (M17) incubated anaerobically for 48 hours at 30°C.

The media will detect contamination based on difference in colony morphologies.

a) The test culture is enriched in RSM, M17 and MRS broth. The tubes are incubated at 37°C for 16 h and then streaked TBA, M17, BCP and MRS agar plates and further incubated at 37°C for 48 h. The enriched culture also is streaked onto KM agar and incubated anaerobically at 30°C for 48 hours.

b) Sign off must be obtained by the person doing quality control (QC) and counter signed by one other starter laboratory person and one production unit person.

c) One vial sent to BLIS Technologies to check for identity using phenotypic (API 20 Strep) and genotyping using ERIC-PCR.

5) When the Production Unit has used a working stock, another seed stock is prepared using the following procedure.

a) The Starter Laboratory will prepare another ~20 vials from Production Unit seed stock as follows:

i) A frozen vial from Production Unit seed stock inoculated into sterile 10% RSM and incubated at 37°C for 16 hours.
ii) Sterile 10% RSM is inoculated with 2% of the overnight culture and incubated at 37°C for 6 hours.
iii) Culture is dispensed into 1.5 mL cryovials and frozen at -80°C.

b) QC is performed as above for master cultures

c) When QC is completed all vials are sent to the Production Unit.

When the Production Unit seed stocks run out, a master vial is used to prepare more seed stock with propagation and QC as for master cultures. The Production Unit seed cultures are kept on a card file system. The file lists the number of vials and their position in the freezer.

Bulk Fermentation

The commercial *S. salivarius* K12 ingredient is prepared as follows:

1) A mixture of demineralized water, skim milk powder, yeast extract, dextrose, and Polysorbate 80 is hydrolyzed with the Neutrase enzyme to form the fermentation media. The media is subsequently heat-treated through ultra-high temperature (UHT) processes, thereby inactivating the enzyme.

2) Inoculum from the Production Unit seed stock is incubated with the media and ammonia (pH control) for 16 h at 33°C and then clarified to produce a cell concentrate.

3) The cell concentrate is mixed with a lyoprotectant (*i.e.,* trehalose, lactitol, maltodextrin, and deionized water), and the pH of the solution is adjusted to 7.3±0.2 using ammonium or sodium hydroxide.

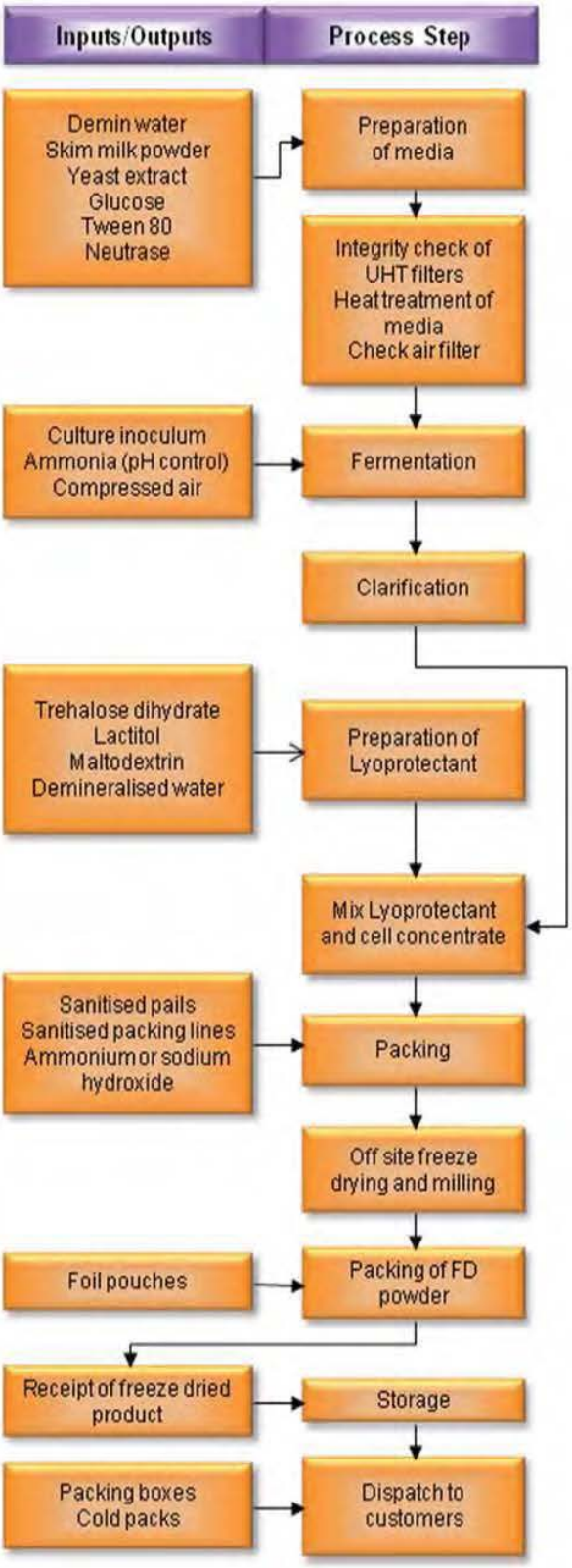
4) The cell concentrate/lyoprotectant solution is sent off-site for freeze-drying and milling.

5) The freeze-dried product is packaged and stored at 2 to 8°C, and is shipped to customers in insulated cartons with cold packs to maintain the chilled conditions.

Samples of the inoculum, sterile fermentation media, sterile lyoprotectant, fermentation broth, cell concentrate and supernatant after centrifugation, product before freeze drying and after milling are obtained and frozen. These samples are analyzed, if the freeze-dried powder is out of specification, to determine the source of contamination. A sample of the inoculum before inoculating the fermenter, and at the end of growth, are tested for optical density (OD), and bacterial morphology checked by a wet mount and Gram stain. If the starter culture is contaminated a new starter is prepared. A representative sample of the freeze-dried powder is taken during packaging at Microbial Fermentation Unit for QC testing in their laboratory. A sample is also sent to BLIS Technologies Research and Development Laboratory to check the identification of *S. salivarius* K12. The powder is rejected if the viable cell count is less than the minimum specifications or if coliforms, *Listeria, Salmonella* and coagulase positive staphylococcus are detected. If the mesophilic aerobic spores, sulfite reducing clostridial spores or yeast and molds are more than twice the allowed specification the powder is rejected, otherwise the powder is held until it can be blended with another batch to bring the product within specification. QC is then conducted on the blended product to ensure it is within specification.

A schematic overview of the manufacturing process of *S. salivarius* K12, including a detailed process description, is presented in Figure II.E-1.

Figure II.E-1 Schematic Overview of the Manufacturing Process for the *Streptococcus salivarius* K12 ingredient



II.F Specifications and Analytical Data for Food-Grade Material

The specifications for the freeze-dried *S. salivarius* K12 powder are presented in Table II-F-1, which include specifications for physical and chemical parameters, as well as specifications for potential chemical and microbiological impurities and contaminants. Details of the methods of analyses for the freeze-dried *S. salivarius* K12 ingredient are provided in Appendices C.

Table II-F-1 Product Specifications for Freeze-Dried *Streptococcus salivarius* K12 Powder

Specification Parameter	Specification	Test Method[a]
Active		
S. salivarius (CFU/g)	NLT $1.0x10^{11}$	BLIS Technologies SOP P011
Physical Properties		
Water activity (a_w)	<0.25	Water activity meter
Particle size	(90) <500 µm	Sieve
Heavy Metals		
Arsenic	NMT 1 ppm	AOAC 935.13 modified/ APHA 3120B Acid dig. ICP-OES
Cadmium	NMT 0.2 ppm	
Lead	NMT 5 ppm	
Mercury	NMT 0.15 ppm	U.S. EPA 245.6 modified
Microbiological Contaminants		
Coliforms (CFU/g)	Not detectable	Compendium 4th Edition 2001
Escherichia coli (CFU/g)	Not detectable	Compendium 4th Edition 2001
Salmonella spp. (CFU/25 g)	Not detectable	ISO 6579:2002 (E)
Aerobic plate count (235°C) (CFU/g)	NMT 200	Compendium 4th Edition 2001
Mesophilic aerobic spores (CFU/g)	NMT 200	Compendium 4th Edition 2001 NZTM2: 59.1
Staphylococcus aureus (CFU/g)	Not detectable	AOAC 2003.07
Molds (CFU/g)	NMT 50	British Pharmacopeia, 2010
Yeast (CFU/g)	NMT 50	British Pharmacopeia, 2010

AOAC = Association of Official Analytical Chemists; APHA = American Public Health Association; CFU = colony forming units; ICP-OES = inductively coupled plasma optical emission spectrometry; NLT = not less than; NMT = not more than; U.S. EPA = United States Environmental Protection Agency; NZTM = New Zealand dairy industry microbiological method

Analyses of 3 non-consecutive lots of the freeze-dried *S. salivarius* K12 powder verify that the material is produced by the manufacturing process in a consistent manner, and complies with the product specifications (Table II.F-2).

Table II.F-2 Analyses for 3 Non-Consecutive Batches of Freeze-Dried *Streptococcus Salivarius* K12 Powder

Specification Parameter	Specification	Manufacturing Lot No.		
		10.02	**10.08**	**11.02**
Active				
S. salivarius (CFU/g)	NLT $1.0x10^{11}$	$1.6x10^{11}$	$1.3x10^{11}$	$1.1x10^{11}$
Identity				
Water activity (a_w)	<0.25	<0.2	<0.2	<0.2
Particle size	$_d$(90) <500 µm	$_d$(90) <500 µm	$_d$(90) <500 µm	$_d$(90) <500 µm
Heavy Metals				
Arsenic	NMT 1 ppm	<1	<0.9	<1
Cadmium	NMT 0.2 ppm	<0.05	<0.05	<0.05
Lead	NMT 5 ppm	<0.3	<0.3	<0.3
Mercury	NMT 0.15 ppm	<0.05	<0.05	<0.05
Microbiological Contaminants				
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
Aerobic plate count (235°C) (CFU/g)	NMT 200	<100	<100	<100
Mesophilic aerobic spores (CFU/g)	NMT 200	<1[a]	<10	<10
Staphylococcus aureus (CFU/g)	Not detectable	<10	<10	<10
Molds (CFU/g)	NMT 50	<10	<10	<10
Yeast (CFU/g)	NMT 50	<10	<10	<10

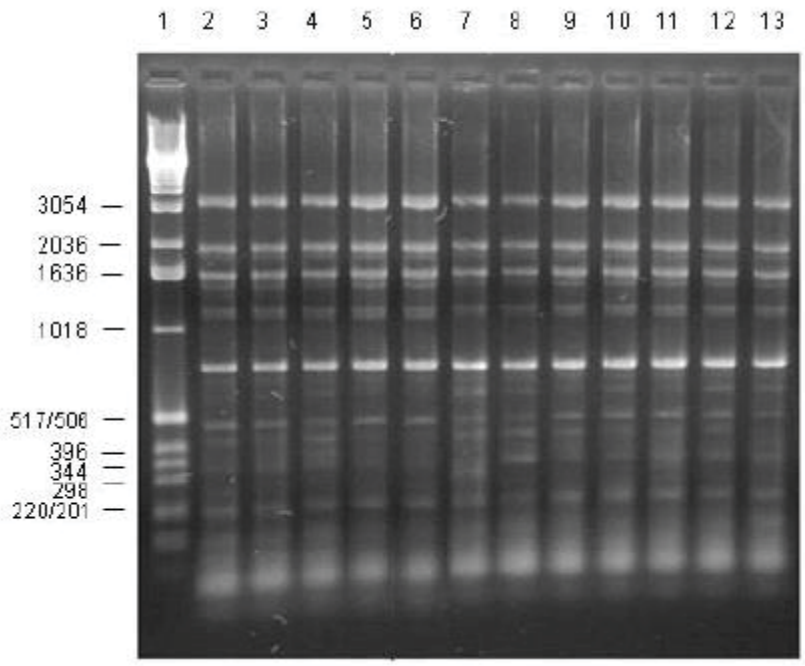
All tests performed by third party accredited facilities
CFU = colony-forming unit; ND = not detected; NLT = not less than; NMT = no more than
[a] Result obtained using method New Zealand dairy industry microbiological method NZTM2:59.1

II.G Stability of the Freeze-Dried *S. salivarius* K12 Powder

1. Genetic Stability of *Streptococcus salivarius* K12

The original master and working cultures for use in production of *S. salivarius* K12 were prepared in November 2000. A historical comparison of batches obtained from several working cultures are presented in Figure II.G-1. Batches 3 through 15 were prepared from working cultures in November 2000, while Batches 19 through 21 were obtained from a new master and working cultures in August 2003. As shown in Figure II.G.1-1, there was no difference in the ERIC-PCR profiles among the commercial batches of freeze-dried *S. salivarius* K12 powder, thus demonstrating that the genetic stability and integrity of the organism is maintained over time.

Figure II.G-1 ERIC-PCR profile of *Streptococcus salivarius* K12 Freeze-Dried Powders



Lane 1 DNA molecular weight marker; Lanes 2-13 batch numbers 3, 4, 5, 7, 8, 9, 10, 12, 14, 15, 18 and 21, respectively.

Corresponding phenotypic analyses of 12 commercial batches of freeze-dried *S. salivarius* K12 powder were conducted, and the analytical results indicate there were no differences in API 20 Strep and API 50CH profiles among the batches (see Tables II.G-1 and II.G-2).

Table II.G-1 API 20 Strep Reactions for Freeze-Dried *Streptococcus salivarius* K12 Powder

Reactions	Batch No.											
	3	4	5	7	8	9	10	12	14	15	18	21
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	-	-	-	-	-	-	-	-	-	-	-	-

Table II.G-1 API 20 Strep Reactions for Freeze-Dried *Streptococcus salivarius* K12 Powder

Reactions	Batch No.											
	3	4	5	7	8	9	10	12	14	15	18	21
D-mannitol	-	-	-	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
D-lactose	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	+	+	+	+	+	+	+	+	+	+	+	+
D-raffinose	+	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-
β-Hemolysis	-	-	-	-	-	-	-	-	-	-	-	-

Table II.G-2 API 50 CH Fermentations for Freeze-Dried *Streptococcus salivarius* K12 Powder

Reactions	Batch No.											
	3	4	5	7	8	9	10	12	14	15	18	21
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-
D-ribose	-	-	-	-	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopranoside	-	-	-	-	-	-	-	-	-	-	-	-
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-
Dulctiol	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-αD-mannopyranoside	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-αD-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-

Table II.G-2 API 50 CH Fermentations for Freeze-Dried *Streptococcus salivarius* K12 Powder

Reactions	Batch No.											
	3	4	5	7	8	9	10	12	14	15	18	21
Arbutine	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	+	+	+	+	+	+	+
D-maltose	+	+	+	+	+	+	+	+	+	+	+	+
D-lactose	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-
D-saccharose	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	+	+	+	+	+	+	+	+	+	+	+	+
D-melezitose	-	-	-	-	-	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+	+	+	+	+	+
Amidon	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	-	-	-	-	-	-
D-tagatose	+	+	+	+	+	+	+	+	+	+	+	+
D-fucose	-	-	-	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-
2-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-
5-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-

Additional testing was conducted to compare the phenotypic profile of a commercial batch of *S. salivarius* K12 (K12-BN21) with the original frozen culture (K12-J89), and a laboratory stock culture (K12-lab), which has been sub-cultured on blood agar every 2 weeks for 3 years. As shown in Tables II.G-3 and II.G-4, the analytical results indicate that metabolic profiles of *S. salivarius* K12 following either recurrent propagation or commercial processing were identical to that of the original isolate, which demonstrates the stable characteristics of this strain (Burton *et al.*, 2006a).

Table II.G-3 API 20 Strep Reactions for *Streptococcus salivarius* K12 Cultures

Reactions	*S. salivarius* K12 culture		
	K12-J89[a]	K12-lab[b]	K12-BN21[c]
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	-	-	-

[a] Original isolate (stored at -70°C for 15 years)
[b] Routinely sub-cultured laboratory isolate (sub-cultured every 2 weeks for 3 years)
[c] A commercially prepared batch of freeze-dried cells

Table II.G-4 API 50 CH Fermentations for *Streptococcus salivarius* K12 Cultures

Reactions	*S. salivarius* K12 culture		
	K12-J89[a]	K12-lab[b]	K12-BN21[c]
Glycerol	-	-	-
Erythritol	-	-	-
D-arabinose	-	-	-
L-arabinose	-	-	-
D-ribose	-	-	-
D-xylose	-	-	-
L-xylose	-	-	-
D-adonitol	-	-	-
Methyl-βD-xylopranoside	-	-	-
D-galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+

Table II.G-4 API 50 CH Fermentations for *Streptococcus salivarius* K12 Cultures

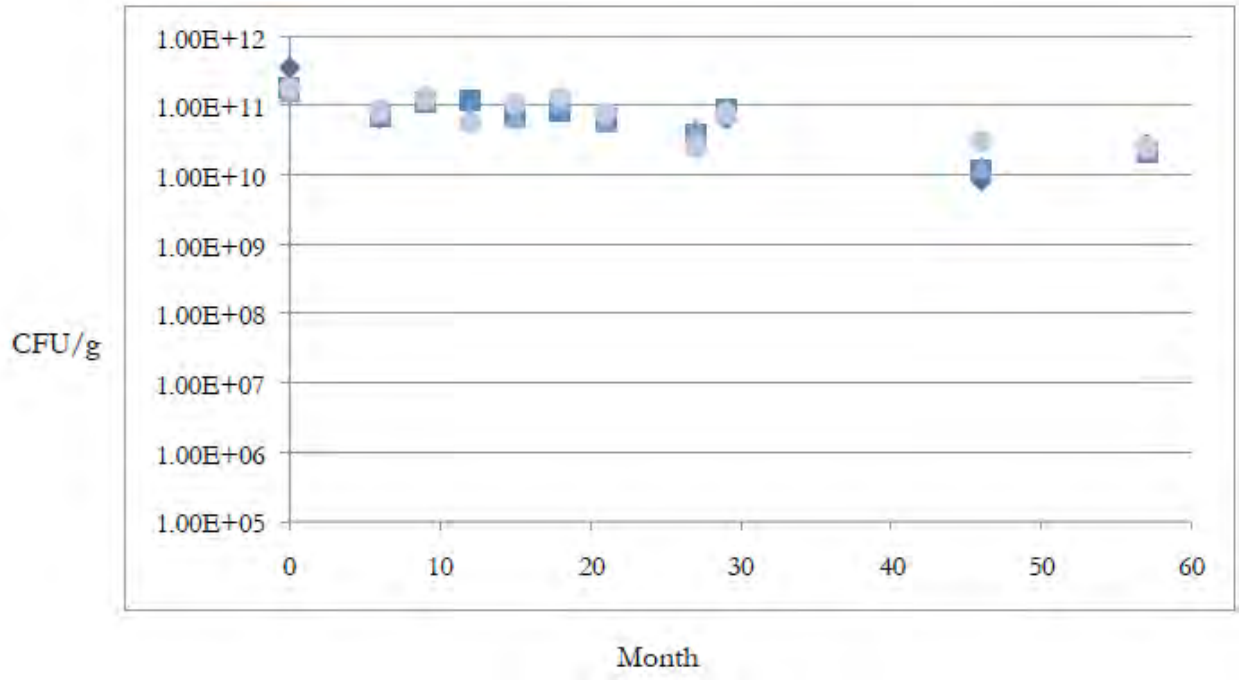
Reactions	*S. salivarius* K12 culture		
	K12-J89[a]	K12-lab[b]	K12-BN21[c]
D-mannose	+	+	+
L-sorbose	-	-	-
L-rhamnose	-	-	-
Dulctiol	-	-	-
Inositol	-	-	-
D-mannitol	-	-	-
D-sorbitol	-	-	-
Methyl-α-D-mannopyranoside	-	-	-
Methyl-α-D-glucopyranoside	-	-	-
N-acetylglucosamine	+	+	+
Amygdalin	-	-	-
Arbutine	+	+	+
Esculin	-	-	-
Salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose	+	+	+
D-melibiose	-	-	-
D-saccharose	+	+	+
D-trehalose	+	+	+
Inulin	+	+	+
D-melezitose	-	-	-
D-raffinose	+	+	+
Amidon	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	-	-	-
D-turanose	-	-	-
D-xylose	-	-	-
D-tagatose	+	+	+
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
Gluconate	-	-	-
2-ketogluconate	-	-	-
5-ketogluconate	-		

[a] Original isolate (stored at -70°C for 15 years)
[b] Routinely sub-cultured laboratory isolate (sub-cultured every 2 weeks for 3 years)
[c] A commercially prepared batch of freeze-dried cells

2. Viability Under Storage Conditions

The stability of freeze-dried *S. salivarius* K12 powder (Batch No. 21) manufactured in late 2003 was measured. Cell counts were conducted over a period of 60 months, and as shown in Figure II.G-2, the cell count loss between the start and end of this period was less than 1 logarithmic unit. The results of this study suggest that freeze-dried *S. salivarius* K12 powder, if stored under the recommended conditions, is stable for at least 3 years at 4°C.

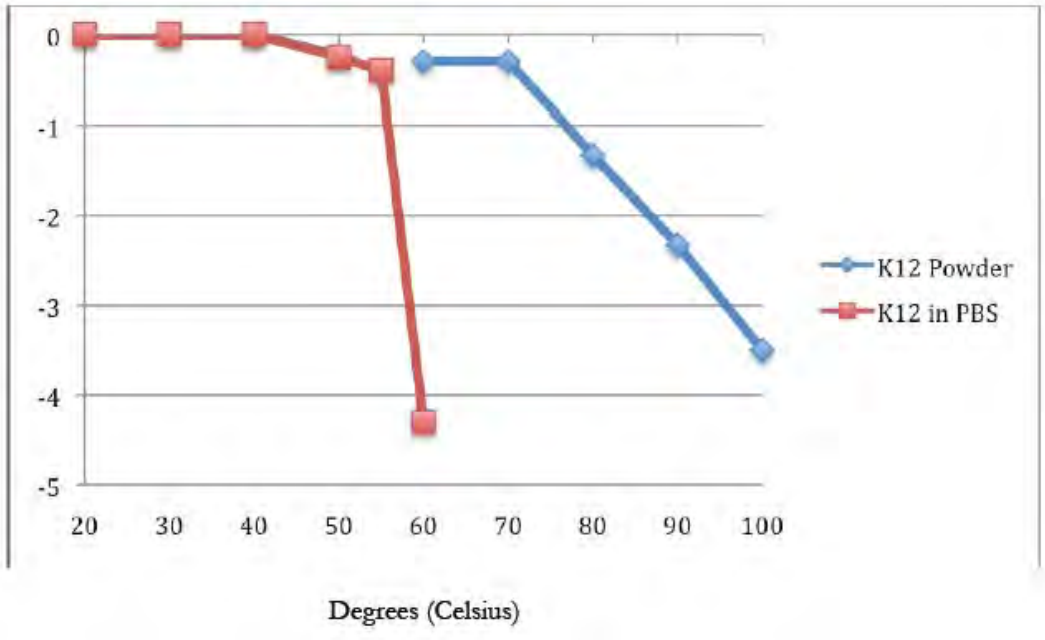
Figure II.G-2 Stability of *Streptococcus salivarius* K12 Lyophilized Commercial Product Stored at 4°C for 60 Months



3. Stability in Food Matrices and Processing Conditions

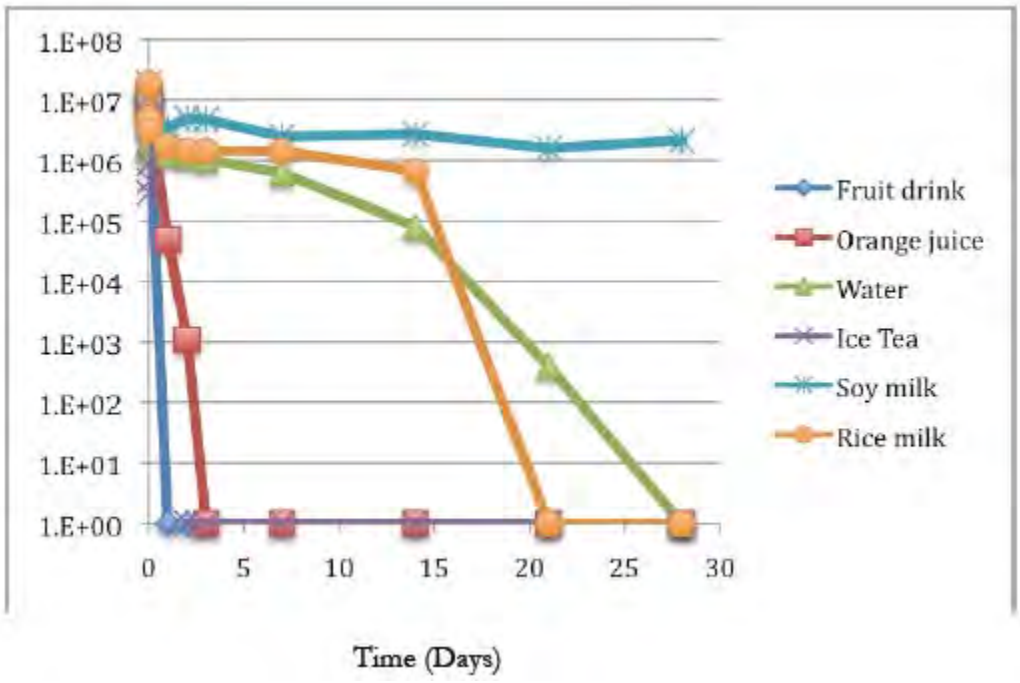
The stability of *S. salivarius* K12 in several food matrices and under different processing conditions has been evaluated. As a lyophilized powder, the organism is moderately stable at elevated temperatures. Over a 5-minute period, good stability was observed at 70°C, with viability of the organism significantly decreasing by several logarithmic units as the temperature is elevated to 100°C, as shown in Figure II.G-3.

Figure II.G-3 Thermal Stability (log CFU decrease) of *Streptococcus salivarius* K12 when Held at Temperature for 5 Minutes



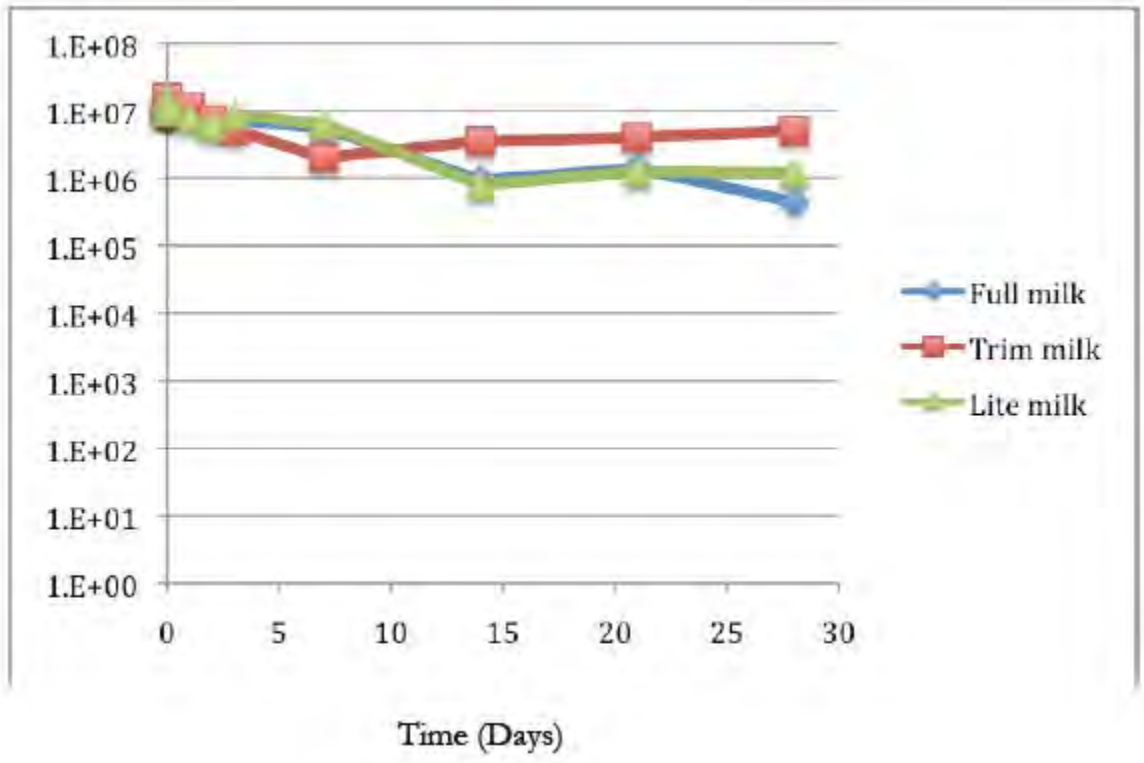
The long-term survival of the organism in various beverage applications (*e.g.*, milk, water and fruit juices) also has been assessed, as shown in Figure II.G-4. At 4°C, *S. salivarius* K12 was shown to be stable in soy milk, and no appreciable loss in CFU count observed over the 25-day storage period. The organism was less stable in rice milk and water, with significant reductions in viability occurring after 2 weeks of storage at 4°C. Long-term *S. salivarius* K12 added to fruit juices and iced tea was poor.

Figure II.G-4 *Streptococcus salivarius* K12 Survival (log CFU) in Various Beverage Types Stored at 4°C



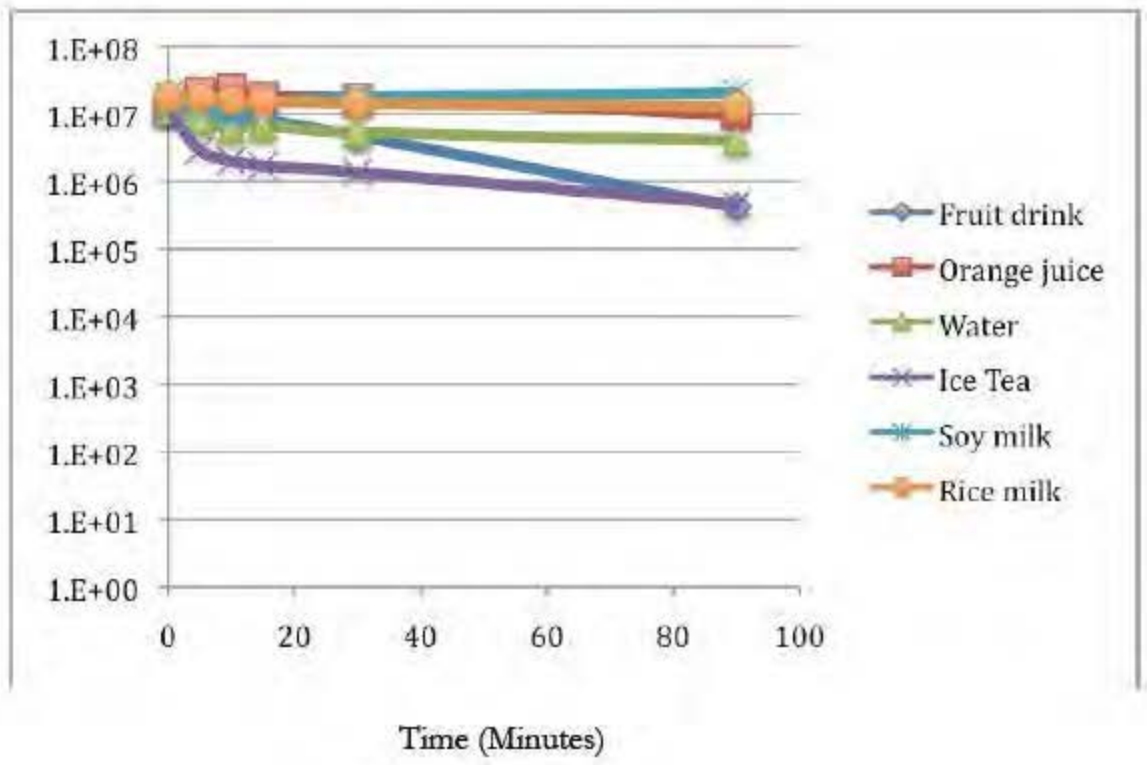
Due to the limited stability of *S. salivarius* K12 in some food types, and the desire to use the organism in these types of foods as reflected in the intended uses included in Table I.D-1, the use of *S. salivarius* K12 in these food types may be achieved through the use with novel delivery systems (release caps and straws). As shown in Figures II.G-5 and II.G-6, suitable short-viability can be achieved food uses that are not amenable to long-term storage of the organism.

Figure II.G-5 Viability of *Streptococcus salivarius* K12 (log CFU) in Various Milks Stored at 4°C



Stored at 4°C in full fat milk (full milk) pH 6.80; low fat milk (Lite milk) 98.5% fat free milk pH 6.83; skim milk (trim) 99.5%; and fat-free milk pH 6.77.

Figure II.G-6 *Streptococcus salivarius* K12 Survival (log CFU) in Various Beverage Types Stored at Room Temperature



III. SELF-LIMITING LEVELS OF USE

No self-limiting levels of use were identified for the *S. salivarius* K12 ingredient.

IV. DETAILED SUMMARY OF THE BASIS FOR THE NOTIFIER'S GRAS DETERMINATION

BLIS Technologies has determined that freeze dried *S. salivarius* K12 powder is GRAS for use in conventional food and beverage products. This GRAS determination was based on scientific procedures using generally available data and information, both favorable and unfavorable, relevant to the safety of the organism for food use. *Streptococcus salivarius* K12 originated from a saliva sample obtained from a healthy child residing in Dunedin, New Zealand. The species *S. salivarius* is a dominant species within the oral cavity (especially on the tongue) of all humans and is present within human milk from lactating women. *S. salivarius* also has a history of food use as a fermentation organism for the manufacture of dairy products. The genetically related starter culture, *S. thermophilus*, has a long-history of wide-spread and safe use as a starter culture for yogurt production. Based on the established and well documented history of consumption of the organism by humans, and the close genetic relation between *S. salivarius* and *S. thermophilus*, the long-history of safe consumption of enumerate strains of the species in the diet throughout the history of mankind provide strong support for the safety of *S. salivarius* K12. Studies summarizing the history of use of *S. salivarius* species are presented in Section IV.A.

Dietary intake of *S. salivarius* K12 was estimated using the intended food uses and use-levels in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES). The estimated intake of *S. salivarius* K12, even among frequent consumers of products which may potentially contain *S. salivarius* K12, were quantitatively comparable with that occurring on a daily basis in all humans from consumption of saliva (Section IV.A).

Acute and subchronic toxicity studies conducted in Sprague-Dawley rats is presented in Section IV.C. *Streptococcus salivarius* K12 also was demonstrated not be genotoxic in the Ames assay using *Salmonella* tester strains TA98, TA100, TA102, TA1535 and TA1537.

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). A similar viewpoint was recently emphasized by a panel of experts who stated that "*for the safety related endpoints important in assessment of probiotics, validated animal models do not exist and, as a result, the determination of safety rests primary on human studies*." (Shane *et al*., 2010). Accordingly, the safety of *S. salivarius* K12 for use in food was based on

information derived from placebo controlled studies in humans evaluating established safety endpoints. Although a recognized limitation to human safety studies relates to their shorter duration, this limitation is not relevant to the safety assessment of *S. salivarius* K12 as the species *S. salivarius* has an established long-history of safe consumption by humans of all ages and demographic types. Safety studies of *S. salivarius* in healthy adults, children and infants are presented in Section IV.D.

As discussed, phylogenetic characterization of *S. salivarius* sp. is sufficiently robust to conclude that the species does not exhibit pathogenic traits of virulent members of the genus. Nevertheless, the pathogenic risk was further characterized, particularly in light of case-reports of iatrogenic infections associated with the species. A comprehensive review of published case-reports and reviews related to incidences of confirmed or suspected *S. salivarius* infection is discussed in Section IV.E. Comprehensive biochemical and bioinformatic evaluations of *S. salivarius* K12 also were conducted confirming the absence of established virulence determinants of *Streptococcus sp*. It was determined that case-reports of established *S. salivarius* infection were almost exclusively limited to iatrogenic circumstances by which exposure of the organism to the cerebrospinal fluid occurred under instances where physicians failed to follow proper hygiene during spinal anesthesia procedures. This route of exposure was not considered relevant to oral consumption of *S. salivarius*. An independent opinion from an expert in pediatric care and Streptococcal disease further corroborate the conclusion that *S. salivarius* K12 does not present a pathogenic risk which differs from that associated with other common lactic acid bacteria used in food for which various cases of opportunistic infection are well established.

Information characterizing the antibiotic resistance of the organism is presented in Section IV.E, and other metabolic features (*e.g*., production of antimicrobials) of the organism were considered. The results of a bioinformatic assessment and comparison of the genome of *S. salivarius* K12 to that of an *S. thermophilus* isolate are presented in Section IV.F and a discussion of post-market surveillance data is presented in Section IV.G.

Finally, the information presented herein was reviewed by the Expert Panel, qualified by scientific training and experience to evaluate the safety of ingredients as components of food. A discussion of the data and information reviewed by the Expert Panel, and conclusion supporting that the intended use of *S. salivarius* K12 in food and beverage products described in Table I.D-1 is included in Appendix A.

IV.A Consumption Estimates

1. History of Safe Use

1.1 Natural Occurrence of Streptococcus salivarius

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 two days after birth. The levels of *S. salivarius* in swab samples taken from newborn infants represent 10% of the total *Streptococci* isolated, increasing to 25 to 30% by 1 month of age (Hegde 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/mL. The total saliva volume produced per day is approximately 500 mL for children (Watanabe *et al.,* 1995) and adults (Porter *et al.,* 2004); therefore, the daily consumption of commensal *S. salivarius* in humans is estimated to range from $5x10^9$ to $5x10^{10}$ CFU/day.

Commensal *Staphylococcus* and Streptococci 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 a number of 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 human breast milk, *S. salivarius* isolates have been identified in fecal samples of infants within the first 3 days, and were a predominant species throughout the breast feeding period (Favier *et al.*, 2002; Park *et al.*, 2005). Viridians Streptococci have been shown to inhibit oral colonization by methicillin-resistant *S. aureus* in infants, and similar inhibitory activity against *S. aureus* growth has been observed with *S. salivarius* isolates obtained from breast milk. Finally, Kirjavainen *et al.* (2001) have noted that the presence of viridians Streptococci was a common feature of the healthy infant gut, in contrast to that observed among atopic infants. The widespread presence and early colonization of *S. salivarius* in the oral cavity, transport through the 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 suggest that the species may play an unappreciated nutritional role in human biology that requires further investigation.

1.2 History of Food Use

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).

2. Estimated Consumption of *S. salivarius* K12 from Intended Food Uses

Estimates for the intake of freeze-dried *S. salivarius* K12 powder was based on the intended food uses and use-levels (Table I.D-1) in conjunction with food consumption data included in the NCHS NHANES for the years 2003-2004 (NHANES 2003-2004) and 2005-2006 (NHANES 2005-2006) (CDC, 2006; USDA, 2009) and grouped in food-use categories according to Title 21, Section § 170.3 of the CFR (U.S. FDA, 2014). Use-levels were calculated based on the amount of freeze-dried *S. salivarius* K12 powder added to a serving of each individual food. Product-specific adjustment factors were developed based on data provided in the standard recipe file for the Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, 1998 survey (USDA, 2000).

Calculations for the mean and 90th percentile all-person and all-user intakes, and percent consuming were performed for each of the individual proposed food-uses of *S. salivarius* K12. Similar calculations were used to determine the estimated total intake of freeze dried *S. salivarius* K12 resulting from all proposed food-uses of *S. salivarius* K12 combined. In both cases, the per person and per kilogram body weight intakes were reported for the following population groups:

- infants, ages 0 to 2;
- children, ages 3 to 11;
- female teenagers, ages 12 to 19;
- male teenagers, ages 12 to 19;
- female adults, ages 20 and up;
- male adults, ages 20 and up; and
- total population (all age and gender groups combined).

It should be noted that this type of intake methodology is generally considered to be “worst case” as a result 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.

2.1 Estimated Daily Intake of S. salivarius K12 from Intended Food Uses

Estimates for the total daily intake of *S. salivarius* K12 resulting from all proposed food-uses on an absolute basis are provided in Tables IV.A-1 and IV.A-2 on a CFU/day and a per kilogram body weight basis.

Approximately 96.9% of the total U.S. population was identified as potential consumers of *S. salivarius* K12 from proposed food-uses (16,173 actual users identified). A high percentage of users were identified in each of the individual population groups (90.0 to 99.8%). As a result of the high percentage of users identified within all population groups, the intake estimates for the all-person and all-user categories were similar; therefore, only the all-user results are discussed in detail. Consumption of proposed food-uses by the total population resulted in an estimated mean all-user intake of *S. salivarius* K12 of $9.8x10^9$ CFU/person/day, which is equivalent to $2.0x10^8$ CFU/kg body weight/day. The 90^{th} percentile all-user intake of *S. salivarius* K12 from proposed food-uses by the total population was $1.9x10^{10}$ CFU/person/day, which corresponds to $4.5x10^8$ CFU/kg body weight/day.

On an individual population basis, the greatest mean all-user intake of *S. salivarius* K12 on an absolute basis was determined to occur in male teenagers at $1.2x10^{10}$ CFU/person/day. Female adults had the lowest mean all-user intake of *S. salivarius* K12 on an absolute basis with a value of $8.6x10^9$ CFU/person/day. On a body weight basis, the mean all-user intake of *S. salivarius* K12 was highest in infants, with an intake of $8.9x10^8$ CFU/kg body weight/day. The lowest all-user mean intakes on a per kilogram body weight basis were observed to occur in female and male adults with a value of $1.2x10^8$ CFU/kg body weight/day, for both population groups.

Table IV.A-1 Summary of the Estimated Daily Intake of *Streptococcus salivarius* K12 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	90^{th} Percentile	Mean	90^{th} 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

When high consumers (*i.e*., intakes at the 90th percentile) were examined, the estimate for the all-user intake of *S. salivarius* K12 from proposed food-uses also was determined to be greatest in male teenagers at 2.3x10^{10} CFU/person/day. The lowest 90th percentile all-user intake estimate was observed to occur in infants with a value of 1.7x10^{10} CFU/person/day, on an absolute basis. On a body weight basis, infants were determined to have the greatest all-user 90th percentile intake of *S. salivarius* K12, with a value of 1.5x10^{9} CFU/kg body weight/day. The lowest all-user 90th percentile intakes of *S. salivarius* K12 on a body weight basis were observed to occur in male adults at 2.4x10^{8} CFU/kg body weight/day.

Table IV.A-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of *Streptococcus salivarius* K12 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/kg bw)		All User (CFU/kg bw)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	90.0	1,722	8.2x10^{8}	1.5x10^{9}	8.9x10^{8}	1.5x10^{9}
Children	3 to 11	99.8	2,728	4.3x10^{8}	7.7x10^{8}	4.3x10^{8}	7.7x10^{8}
Female Teenagers	12 to 19	98.8	1,964	1.6x10^{8}	3.4x10^{8}	1.7x10^{8}	3.4x10^{8}
Male Teenagers	12 to 19	98.1	1,903	1.9x10^{8}	3.7x10^{8}	1.9x10^{8}	3.7x10^{8}
Female Adults	20 and up	97.3	4,164	1.2x10^{8}	2.4x10^{8}	1.2x10^{8}	2.5x10^{8}
Male Adults	20 and up	96.1	3,692	1.1x10^{8}	2.4x10^{8}	1.2x10^{8}	2.4x10^{8}
Total Population	All Ages	96.9	16,173	1.9x10^{8}	4.4x10^{8}	2.0x10^{8}	4.5x10^{8}

bw = body weight; CFU = colony forming units

IV.B Colonization and Metabolic Fate

1. Gastrointestinal Tract

There is limited information characterizing colonization and metabolic fate of *S. salivarius* K12 following oral consumption by humans or animals. 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 gavaged with a microorganism mixture containing *L. 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, 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 mucus 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." As discussed, the species *S. salivarius* is specific to humans and findings in

rodent studies are therefore of unclear relevance to humans. Nevertheless, the species *S. salivarius* displays poor acid tolerance (Chen *et al.*, 2000), and other phenotypes common to commensal microorganisms within the gastrointestinal tract of animals, including active bile-salt hydrolase activity, and resistance to digestive enzymes have not been demonstrated for the species. Microorganisms are adapted for survival within specific niches to which they have become adapted, and active colonization of *S. salivarius* within the gastrointestinal tract of humans is not expected. Consistent with the conclusion Streptococcal species constitute a minor fraction of the gut microflora (Benson *et al.*, 2010; Arumugam *et al.*, 2011). Accordingly, consumption of *S. salivarius* K12 in the diet is not expected to effect the "normal" microflora 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.

2. Colonization and Effects on Oral Microflora

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). Oral colonization sites for *S. salivarius* species include the pharynx, tongue and buccal membrane. Burton *et al.* (2006b) measured the colonization of *S. salivarius* K12 in a group of 23 healthy subjects (10 female, 13 male, average age of 43.3 years). Subjects were randomized to 1 of 2 groups administered a *S. salivarius* K12 lozenge containing $>1x10^9$ CFU/lozenge or a placebo lozenge every 2 hours over an 8-hour duration on days 1, 2, and 3. To improve colonization with the organism, all subjects undertook mechanical and chemical oral cleansing *via* toothbrushing, tongue scraping and use of antibacterial mouthwash on each day. After the 3-day colonization period *S. salivarius* K12 or placebo lozenges were administered to each individual, after chemical and mechanical oral cleansing once in the morning and once before bedtime, for a duration of 2 weeks. Microbiological analyses were conducted at baseline and on days 7 and 14 using culture methods and DGGE. The DGGE fragment patterns corresponding to *Prevotella veroralis*, *P. melaninogenica* and *Veillonella dispar* predominated in the subjects. *Streptococcus salivarius* K12 could be detected in the saliva samples of all subjects administered *S. salivarius* K12 containing lozenges; however, *S. salivarius* K12 consumption did not affect total microflora counts of *S. salivarius*. On days 7 and 14 differences in the DGGE profiles of saliva samples could be observed relative to baseline in some subjects administered *S. salivarius* K12 suggestive that changes in the microflora populations had occurred in these individuals. The DGGE profiles measured represented only a fraction of the total microflora population within the oral cavity, and although quantitative changes in total microflora populations following administration of *S. salivarius* K12 could not be determined, using *in vitro* studies the authors

demonstrated that bacteriocins produced by *S. salivarius* K12 are inhibitory towards various 'undesirable' commensal species within the oral cavity including *Streptococcus anginosis*, *Eubacterium saburreum* and *Micromonas gingivalis*. It is noteworthy that changes in the DGGE profiles reported by the authors were not permanent and rapid reversion to pre-treatment profiles were observed following discontinuation of *S. salivarius* consumption.

Using the same colonization procedure to that described by Burton *et al.* (2006b), Horz *et al*. (2007) evaluated the colonization of *S. salivarius* K12 in a single subject. The authors reported that "*the introduction of K12 (four times a day for 3 days) into the oral cavity does not necessarily result in high numbers or long-term colonization of K12*". In addition, the authors also noted that colonization of the oral cavity was limited to saliva and mucosal membranes with colonization absent the gingival sulci and teeth of the individual. Similar observations demonstrating transient non-permanent colonization of *S. salivarius* K12 were observed by Burton *et al*. (2011) who reported that daily consumption of *S. salivarius* K12 ($1x10^{10}$ CFU/ person/day) formulated into a drink for a period of 28 days produced a 5-fold increase in *S. salivarius* counts that reverted to normal at the end of the wash-out period. Investigations conducted by Power *et al*. (2008) whereby 19 young otitis media prone children were administered *S. salivarius* K12 twice daily for 10 days (total daily dose of $2.0x10^{7}$ to $1.0x10^{10}$ CFU/child/day) after amoxicillin treatment further demonstrate that colonization of the oral cavity by probiotic organisms is limited and subject to significant inter-individual variability with some individuals highly resistant to colonization.

Findings discussed above demonstrate that the consumption of *S. salivarius* K12 from intended food uses will not result in permanent colonization of the oral cavity of consumers, nor are permanent/significant changes in the overall oral microflora composition expected. The effects of consuming *S. salivarius* K12 in the diet on the microflora composition of the gastrointestinal tract are comparable to those observed following the consumption of other GRAS microorganisms in the diet such as lactobacillus and bifidobacterium species, which also have demonstrated to result transient residence in the gastrointestinal tract following discontinuation of repeated consumption in the diet.

IV.C Toxicity of *S. salivarius* K12

1. Acute Toxicity

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 Organization of Economic Co-operation and Development (OECD) Guideline No. 407 (OECD, 2008). Fifty-nine rats (59) were randomized into 5 groups. Groups 1, 2, and 3 were administered either 1 of 3 bolus doses of the freeze-dried *S. salivarius* K12 powder (providing $1.25x10^{8}$, $1.67x10^{9}$, or $8x10^{10}$ CFU of

S. salivarius K12/rat). The 3 doses of *S. salivarius* K12 were 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) or sterile saline. One rat from each group was killed 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 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. There were no signs of tissue abnormalities reported in rats administered *S. salivarius* K12. A blood sample taken on day 14 from a mid-dose male identified a positive culture in Mitis salivarius agar*;* however, further testing of the DNA fingerprint of this organism indicated that the isolate was not *S. salivarius* K12. The investigators suggested that contamination with an organism naturally present in the animal may have occurred due to perforation of the esophagus during intra-cardiac bleeding at time of termination. Based on their findings, 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, the highest dose tested, when orally administered to rats.

2. Sub-Acute Toxicity Studies

The toxicity of *S. salivarius* K12 has been investigated in rodents (Burton *et al.*, 2010). Mature Sprague-Dawley rats (20/sex/group) were fed diets providing 7.5, 100, or 5,000 mg/kg body weight/day of freeze-dried *S. salivarius* K12 powder for a period of 28 days; these doses provided a corresponding intake of $1.25x10^9$, $1.67x10^9$, or $8x10^{10}$ CFU per kg body weight respectively. An additional group of rats was fed a diet providing 7.5 mg lycoprotectant/kg body weight/day. Five animals of each sex were killed at the beginning of the study to provide baseline parameters for all endpoints. Blood samples were taken on days 1, 7, 14, 21, and 28 and analyzed for serum biochemistry[2] and hematology[3] parameters. Analyses of blood samples for bacterial translocation also were conducted. On day 28, 10 animals/sex/ group were necropsied, while the remaining animals were monitored for an additional 28-day recovery period. On days 28 and 56, urine samples were collected and urinalysis[4] was conducted.

S. salivarius K*12* treatment was well-tolerated by the animals and the authors' reported that "…*none of the test or control groups of animals demonstrated any detectable health change throughout the 28-day test period.*" No adverse effects on general clinical signs, ophthalmologic

[2] Parameters evaluated included aspartate aminotransferase (AST), alanine aminotransferase (ALP), total protein, creatinine, urea, total bilirubin, albumin, cholesterol, glucose, and triglycerides.
[3] Parameters evaluated included hematocrit, hemoglobin, mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell, white blood cell, lymphocyte, monocyte, eosinophil, neutrophil, and reticulocyte counts
[4] Parameters evaluated included color/turbidity, urobilinogen, occult blood, bilirubin, specific gravity, creatinine, pH, glucose, sediment, protein, ketones, and nitrites.

evaluations, organ weights, or gross pathology were reported in any dose group during the administration period. Body weights were reported to be significantly increased in high-dose males compared to the other male dose groups; 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. The higher weight gain in high-dose males may have been due to the higher caloric intake of maltodextrose (lyoprotectant) by these animals. No differences in body weight were noted among female dose groups. Food consumption was reported to be initially reduced in week 1 in all male dose groups, and in week 2 for all female dose groups; however, the amount of reduced intake was reported to be small in comparison to the normal consumption pattern, and food consumption returned to normal in all groups.

Increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels outside reference control values were reported in all groups at day 56, including control animals. However, based on the age of the animals and the fact that the corresponding baseline measures were similarly elevated outside the reference range demonstrate that the changes were not compound-related, but instead likely related to ageing and fatty changes of the liver. All other serum biochemistry parameters were reported to be within reference or baseline ranges.

Urinalysis did not reveal any significant adverse effects or variations. Animals fed *S. salivarius* K12 were reported to exhibit slightly more acidic urine and slightly higher red blood cell (RBC) counts in the urine; however, the authors considered these findings to be toxicologically insignificant, as they were not accompanied by any other biochemical indicators of kidney damage. Sporadic and minor elevations in other parameters (*i.e.*, mean corpuscular volume, mean corpuscular hemoglobin concentration, and eosinophil count) were reported (group not specified); however, these findings were considered by the investigators to be spontaneous, as they were not associated with elevations in other hematology parameters indicating an adverse effect.

A day 28 blood sample from a high-dose female was reported to be positive for the presence of *S. salivarius* K12. The investigators stated that this occurrence was most likely due to perforation of the esophagus or trachea during intra-cardiac bleeding at time of termination, given that no evidence of endocarditis was identified during necropsy and heart weight was normal. The study investigators concluded that "*No abnormal finding occurred for any of the animals on test. Of the parameters measured, which included haematology, biochemistry, urinalysis, organ weights, body weights, clinical observations, none of these provided any evidence of an adverse effect as a result of being treated by repeated dosing with the test substance at three dosages as a sub-acute administration in feed*." (Goldenthal *et al*., 2005)

Overall, the authors reported no signs of overt toxicity were observed in mature Sprague-Dawley rats orally administered up to 5,000 mg/kg body weight/day ($8x10^{10}$ CFU per kg body weight/day), of the freeze-dried *S. salivarius* K12 powder, for a period of 28 days.

3. Other Animal Studies

Several studies conducted using mice and rats have evaluated the effects of oral administration of *S. salivarius* K12, and other non-related *S. salivarius* strains, on various efficacy related measures. Study designs utilized 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* K12. These studies are presented below for completeness. Overall, none of the study presented findings to suggest that the intended use of *S. salivarius* K12 as a food ingredient may present cause for concern.

3.1 S. salivarius K12

The influence of a multi-probiotic preparation containing *S. salivarius* K12 on sulfasalazine metabolism in the rat was evaluated by Lee *et al.* (2012). Sulfasalazine is a drug that is converted to the active metabolites sulfapyuridne and 5-acetylsalicylic acid by the azoreductase activity of commensal microorganisms within the gastrointestinal tract. Male Wistar rats aged 6 to 8 weeks (n=5 per group) were randomized to 1 of 2 groups administered the following treatments: 1) 2 g of a probiotic preparation containing *L. acidophilus* LAFTI L10 ($4x10^8$ CFU/g), *B. lactis* LAFTI B94 ($4x10^8$ CFU/g), and *S. salivarius* K12 ($1x10^8$ CFU/g); 2) 2 g of probiotic powder excipients (maltodextrin, trehalose, and lactitol). Test powders were dissolved in water and administered twice daily (total daily intake of 4 g/powder/day) for 3 days. Two additional groups of control and probiotic treatment rats (n=8 per group) were administered an oral dose of sulfasalazine (100 mg/kg body weight) in saline *via* gavage 6 hours after completion of the 3-day treatment period. Animals from this group were used to evaluate the pharmacokinetics of sulfasalazine metabolism. Azoreductase activity of the microflora was measured in the remaining animals by *ex-vivo* measurements of the ileal and colon contents of the animals. The authors reported that probiotic treatment increased the azoreductase activity of the colon contents; however, no corresponding influence of sulfasalazine metabolism or pharmacokinetics was observed.

The effect of *S. salivarius* K12 on a rodent oral candidiasis model was reported by Ishijima *et al.* (2012). Female ICR mice (6 weeks of age) inoculated with Candida were randomized to 1 of 5 groups administered 50 µl solutions containing saline (control, n=15,), *S. salivarius* K12 (7.5 mg/mL, n=7); *S. salivarius* K12 (15 mg/mL, n=12), *S. salivarius* K12 (30 mg/mL, n=15), or fluconazole (2 mg/mL, n=6). At 48 hours post-inoculation, each animal was euthanized *via* cervical dislocation and score severity of lesions assessed. Measurements of viable *Candida* cells and histological examinations of the murine tongues also were conducted. No adverse findings attributable to *S. salivarius* K12 treatment were reported by the authors. The authors concluded that *S. salivarius* K12 "*may inhibit the process of invasion of C. albicans into mucous surfaces*." Safety relevant measures were not reported by the authors.

3.2 Non-Related Strains of S. salivarius

Several studies have been conducted to examine the effects of other *S. salivarius* strains in rats (Hamada *et al.*, 1978; Tanzer *et al.*, 1985). No significant effects on body weight gain were reported in rats orally administered up to $6x10^8$ CFU of *S. salivarius* TOVE-R for up to 8 days (Tanzer *et al.*, 1985). In a study conducted to investigate the potential cariogenicity of *S. salivarius* strains HT3R and HT9R, no adverse effects were reported in rats orally inoculated with 8 doses of $1x10^{12}$ CFU/day of bacteria over a period of 11 days (Hamada *et al.*, 1978). No findings were reported by these authors to suggest that administration of *S. salivarius* strains to a foreign host adversely effects oral/dental health, or general wellbeing of the animals. A summary of information reported by the authors is presented in Table VI.C-1.

Table VI.C-1 Summary of Repeated-Dose Studies on Other *Streptococcus salivarius* Strains

Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Test Compound	Dose	Reported Effects [a,b]		
Rat (Osborne-Mendel, sex NR) 9-10/group	Oral (gavage) Two doses	*S. salivarius* TOVE-R (streptomycin-resistant; colonially rough)	$6x10^8$ CFU/dose	General condition/ survival	• Not evaluated	
				Food and water intake	• Not evaluated	
				Body weight	• NSD in body weight gain	
				Organ and tissue effects	• Initial colonization inhibited th emergence of focally transmit mutans *S.* 10449S and 6715	
				Hematology, clinical chemistry, and urinalysis	• Not evaluated	
				NOAEL	Not identified	
Rat (Osborne - Mendel, sex NR) 9-13/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/d	General condition/ survival	• Not evaluated	
				Food and water intake	• Not evaluated	
				Body weight	• NSD in body weight gain.	
				Organ and tissue effects	• TOVE-R colonized the teeth, the tongue • TOVE-R displaced the mutan *Streptococci* on the teeth, whic associated with a significant inhibition of caries induced by *S. mutans* or *S. sobrinus*	
				Hematology, clinical chemistry, and urinalysis	• Not evaluated	
				NOAEL	Not identified	

Table VI.C-1 Summary of Repeated-Dose Studies on Other *Streptococcus salivarius* Strains

Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Test Compound	Dose	Reported Effects [a,b]		
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/d	General condition/ survival	• No adverse effects reported authors.	
				Food and water intake	• Not evaluated	
				Body weight	• Not evaluated	
				Organ and tissue effects	• *S. salivarius* strains were non cariogenic	
				Hematology, clinical chemistry, and urinalysis	• Not evaluated	
				NOAEL	Not identified	

↓ = decrease(d); ↑ = increase(d); ALT = alanine aminotransferase; AST = aspartate aminotransferase; bw = body weight; CFU = colony for
M = male; NOAEL = no-observed-adverse-effect level; NR = not reported; NSD = no significant differences
[a] unless stated otherwise, all reported effects are relative to control group(s)
[b] numbers in [ ] correspond to the dose(s) at which the reported effects were observed

4. Mutagenicity Studies

A bacterial reverse mutation assay (Ames test) was conducted to assess the potential mutagenicity of the freeze-dried *S. salivarius* K12 powder at concentrations of 50, 158, 500, 1,581, or 5,000 µg/plate in *Salmonella* Typhimurium strains TA98, TA100, TA102, TA 1535, and TA1537, with and without metabolic activation (Burton *et al.*, 2010). This study was conducted in accordance with the OECD Principles of Good Laboratory Practices (OECD, 1998). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for assays conducted in the absence of metabolic activation: sodium azide, 2-nitrofluorene, or cumene hydroperoxide. For assays conducted in the presence of metabolic activation, benzo[a]pyrene, cyclophosphamide, or 9-aminoacridine was employed as the positive control. Treatment with the freeze-dried *S. salivarius* K12 powder did not result in a significant increase in the mean number of revertant colonies compared with the negative control at any concentration, with or without metabolic activation. Positive control agents significantly increased the number of revertant colonies compared to the negative control, thus confirming the sensitivity of the assay. Based on these findings, the freeze-dried *S. salivarius* K12 powder is non-mutagenic as assessed in the Ames test.

IV.D. Human Studies

1 Safety Studies in Healthy Adults

The safety and oral tolerance of *S. salivarius* K12 was evaluated by Burton and colleagues (Burton *et al*., 2011). The study was a randomized, double-blind, placebo-controlled, parallel-arm study, conducted in a group of 56 healthy male and female adults. Participants were randomized to groups provided 0 (placebo) or $1.1x10^{10}$ CFU/day of *S. salivarius* K12 for a period of 28 days. The *S. salivarius* K12 cultures were grown in an ISO 22000 (ISO standards for food safety) quality-accredited facility. The powder containing *S. salivarius* K12 was blended with commonly used food-grade carriers (*i.e.*, trehalose, maltodextrin, and flavorings) and packaged into sachets according to Good Manufacturing Practice. Cell counts of the test organism were measured by an independent laboratory at the start of the study. Unused sachets returned at the end of the study were analyzed to confirm the identity and cell counts of the test organism. The placebo sachets did not contain *S. salivarius* K12 powder. Subjects were instructed to consume the probiotic or placebo powders reconstituted in 4 ounces (half a glass) of cold water at breakfast.

Blood samples were collected at screening (week1) and end of treatment (week 4). Serum chemistry and liver function tests included analysis for glucose, blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, calcium, albumin, globulin, bilirubin, alkaline phosphatase, AST, and ALT. Hematology parameters assessed included white blood cell (WBC) count, RBC count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentrations (MCHC),

platelets, neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Urine samples were collected at baseline (week 0) and end of treatment, and evaluated for clarity, color, specific gravity, pH, protein, occult blood, and leukocyte esterase. Non-stimulated saliva samples were obtained at baseline, end of treatment, and follow-up (week 8). Counts of *S. salivarius* K12 in saliva were assessed using real-time PCR and *S. salivarius* K12-specific primers, according to a standard amplification protocol (Hyink *et al.*, 2007). Assessments of oral and gastrointestinal health *via* self-reported questionnaires were conducted at baseline, end of treatment, and follow-up. Subjects rated items related to oral health (*i.e.*, conditions of teeth, mouth, gums, and freshness of breath) and gastrointestinal health (*i.e.*, presence and severity of pain, bloating, gastric reflux, nausea, vomiting, diarrhea, and flatulence) using a 10-point severity visual analogue scale (VAS).

Sixty-seven (67) volunteers underwent initial screening for eligibility, and 56 healthy subjects were subsequently enrolled and randomized to the probiotic (n=27) and placebo (n=29) groups. Baseline characteristics of the 53 subjects who completed the study (25 and 28 in the probiotic and placebo group, respectively) were reported to be comparable between groups.

As summarized in Table IV.D-1, there were no significant between-group differences in change from baseline values for any of the vital signs, clinical chemistry, or hematology parameters assessed. Analysis of the urine samples indicated a significant difference in the change from baseline for specific gravity between the probiotic and placebo groups. The placebo group exhibited a greater increase in specific gravity (*i.e.*, 0.006) compared to the probiotic group (*i.e.*, 0.001); however, end of treatment values for both the probiotic and placebo groups (*i.e.*, 1.016 and 1.022, respectively) were well within the normal range for healthy humans (*i.e.*, 1.005 to 1.030; LabCorp). In addition, there were no significant differences reported between groups in any of the other urinary parameters measured.

Table IV.D-1 Absolute Values for Vital Signs, Serum Chemistry, Hematology, and Urinalysis Parameters

Parameter	Probiotic Group		Placebo Group	
	Baseline	End of Treatment	Baseline	End of Treatment
Vital Signs				
Heart rate (bpm)	71.0 ± 10.2	72.8 ± 10.1	71.2 ± 9.7	72.1 ± 9.2
Systolic blood pressure (mmHg)	117.1 ± 11.0	119.4 ± 12.1	118.0 ± 10.7	118.4 ± 14.1
Diastolic blood pressure (mmHg)	75.9 ± 6.6	77.9 ± 7.3	74.6 ± 7.8	76.9 ± 9.2
Respiration rate (bpm)	14.7 ± 1.8	13.8 ± 1.9	13.8 ± 1.7	14.1 ± 1.8
Oral temperature (°F)	98.0 ± 0.5	98.2 ± 0.7	98.0 ± 0.5	98.2 ± 0.6
Serum Chemistry				
Glucose	89.1 ± 9.0	92.0 ± 8.9	91.5 ± 9.3	92.6 ± 10.5
BUN	12.3 ± 2.8	13.0 ± 3.3	12.3 ± 3.9	12.8 ± 3.8
Creatinine	0.82 ± 0.15	0.82 ± 0.14	0.85 ± 0.15	0.86 ± 0.15

Table IV.D-1 Absolute Values for Vital Signs, Serum Chemistry, Hematology, and Urinalysis Parameters

Parameter	Probiotic Group		Placebo Group	
	Baseline	End of Treatment	Baseline	End of Treatment
Sodium	139.0 ± 2.0	139.2 ± 1.8	139.8 ± 2.4	139.3 ± 2.0
Potassium	4.14 ± 0.25	4.02 ± 0.26	4.10 ± 0.31	4.08 ± 0.36
Chloride	102.8 ± 2.8	102.1 ± 2.0	102.9 ± 1.7	102.6 ± 2.1
Calcium	9.3 ± 0.4	9.2 ± 0.3	9.4 ± 0.3	9.2 ± 0.2
Albumin	4.38 ± 0.34	4.33 ± 0.29	4.36 ± 0.25	4.30 ± 0.23
Globulin	2.63 ± 0.34	2.64 ± 0.34	2.73 ± 0.39	2.70 ± 0.35
Bilirubin	0.49 ± 0.2	0.49 ± 0.3	0.49 ± 0.22	0.45 ± 0.23
Alkaline phosphatase	59.4 ± 12.6	60.5 ± 14.4	66.4 ± 16.0	68.6 ± 24.6
AST	20.9 ± 5.5	21.5 ± 4.9	21.0 ± 9.8	21.8 ± 15.5
ALT	19.0 ± 7.3	18.9 ± 6.9	20.3 ± 13.1	20.9 ± 12.6
Hematology				
WBC count	7.12 ± 1.90	6.83 ± 1.82	6.53 ± 1.86	6.50 ± 2.20
RBC count	4.58 ± 0.37	4.58 ± 0.35	4.72 ± 0.47	4.69 ± 0.43
Hemoglobin	13.9 ± 1.2	13.9 ± 1.3	13.9 ± 1.4	13.9 ± 1.5
Hematocrit	41.9 ± 3.0	41.5 ± 3.1	42.1 ± 3.6	41.4 ± 3.9
MCV	91.7 ± 5.9	90.9 ± 5.6	89.5 ± 5.3	88.4 ± 5.2
MCH	30.5 ± 2.4	30.4 ± 2.2	29.6 ± 2.1	29.7 ± 2.1
MCHC	33.2 ± 1.1	33.5 ± 1.1	33.1 ± 0.8	33.5 ± 0.9
Platelets	291.5 ± 57.9	293.5 ± 72.9	279.1 ± 65.8	284.0 ± 68.8
Neutrophils	58.8 ± 9.0	56.0 ± 8.8	57.5 ± 8.2	54.9 ± 10.5
Lymphocytes	30.6 ± 8.7	32.8 ± 8.4	31.4 ± 7.5	33.6 ± 9.5
Monocytes	7.68 ± 1.97	7.88 ± 2.20	8.54 ± 2.12	8.61 ± 2.45
Eosinophils	2.36 ± 1.93	2.76 ± 2.18	2.00 ± 0.82	2.36 ± 1.28
Basophils	0.56 ± 0.58	0.52 ± 0.59	0.46 ± 0.51	0.54 ± 0.51

ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentrations; MCV = mean corpuscular volume; RBC = red blood cell; WBC = white blood cell.
Values are mean ± SD where applicable.
Differences between the probiotic and placebo groups were assessed by an analysis of covariance that included the effects of treatment group and baseline assessment as a covariate; no statistically significant differences reported (*i.e.*, P>0.05).

Analysis by quantitative PCR indicated that the mean count of *S. salivarius* K12 in saliva increased from $1.84x10^3$ CFU/mL at baseline to $5.24x10^3$ CFU/mL at the end of treatment in the probiotic group (statistical significance not reported). Following the 4-week follow-up period, the mean *S. salivarius* K12 count (*i.e.*, $1.61x10^3$ CFU/mL) was reported to be similar to baseline levels.

Results of the self-reported questionnaires indicated no significant differences between the probiotic and placebo groups with respect to the subjects' perception of the condition of their teeth, freshness of their breath, or the extent of gum bleeding at the end of treatment or follow-up compared to baseline. Tooth sensitivity was reported to be significantly decreased in the probiotic group relative to the placebo group at the end of treatment and follow-up periods; however, the authors noted that these results were not statistically significant once sex distribution was accounted for. There were no statistically significant differences reported between groups with respect to abdominal pain complaints, bloating, gastric reflux, nausea, vomiting, diarrhea, or flatulence throughout the study. The authors reported a significant between-group difference (P=0.006) after the treatment period; subjects in the placebo group reported a decline in their general gastrointestinal health, whereas responses from subjects in the probiotic group remained relatively stable.

No serious adverse events were observed in either group. The majority of non-serious adverse events observed in the probiotic (n=14) and placebo (n=21) groups were reported to be mild in intensity. None were reported as severe in the probiotic group, whereas 2 were of severe intensity in the placebo group. One adverse event, due to lumbar pain from an accidental fall, was not coded in the probiotic group. As indicated in Table IV.D-2, the number of adverse events that coincided with either probiotic or placebo consumption and considered possibly related to the treatments was very low.

Table IV.D-2 Adverse Events Related to Treatment[a]

Adverse Events	Probiotic Group (n=27)	Placebo Group (n=29)
Number of subjects with ≥1 AE	2 (7.4%)	2 (6.9%)
Anxiety	0 (0%)	1 (3.4%)
Dizziness	0 (0%)	1 (3.4%)
Dyspepsia	1 (3.7%)	1 (3.4%)
Flatulence	1 (3.7%)	0 (0%)

AE = adverse event.
[a] Events considered as possibly, probably, or highly probably related to treatment.

2 Other Studies

Several additional studies have been conducted in adult subjects evaluating various beneficial effects of consuming *S. salivarius* K12 in the diet. Although various limitations in the methodology of these studies limit their use in the safety assessment, the results of these studies consistently demonstrate that *S. salivarius* K12 is well tolerated and without adverse effects, and therefore corroborate the safety of ingredient. Safety and tolerance measures obtained from the large placebo controlled trial conducted in infants consuming *S. salivarius* K12 supplemented infant formula on a daily basis for 12 months also provide strong supportive evidence that *S. salivarius* K12 is expected to be safe and well tolerated across all age groups. These studies are discussed further in Sections 2.1 through 2.3 below.

2.1 Studies Conducted in Adults

The administration of slow release tablets containing *S. salivarius* K12 freeze-dried powder to a group of 40 male and female adults with diagnosis of recurrent oral *Streptococcal* pharyngitis was evaluated by Di Pierro *et al*. (2013). Subjects recruited to the study were between the ages of 18 and 65 years with diagnosis of recurrent (4 episodes) group A hemolytic *Streptococcus* pharyngitis and/or tonsillitis in the previous year. All subjects displayed a total absence of symptoms of infective disease at the time of enrolment. Participants randomized to the treatment group (n=20, aged 33.0±6.4 years) were administered a single slow release tablet containing $5x10^9$ CFU *S. salivarius* K12 once daily before bedtime after oral cleansing (teethbrushing and/or mouthwash use) for a period of 90 days. The remaining subjects (n=20, aged 35.7±7.0 years) served as the untreated concurrent control group. At the end of the 90-day treatment period 16 subjects from the treatment group and 17 of the non-treated subjects were followed for an additional 6 months during which time the product was not administered. Clinic visits were conducted every 15 days. When oral pathologies occurred participants reported directly to the clinic without regard to scheduling. Tolerability, compliance, and side-effects were evaluated as secondary measures during the 90-day treatment period.

Lee *et al.* (2010) conducted a preliminary study to assess the effects of a combination of probiotics on sulfasalazine metabolism in individuals with rheumatoid arthritis. Twelve patients ingested a probiotic preparation providing $2x10^8$ CFU/day of *S. salivarius* K12, $8x10^8$ CFU/day of *Lactobacillus acidophilus* L10, and $4x10^8$ of *Bifidobacterium lactis* B94 for a period of 1 week. Blood and urine samples were taken before and after probiotic treatment, and after a 3-week washout period. No significant changes in full blood count or liver and function tests were reported during the treatment period. Three patients reported gastrointestinal disturbances and 1 patient reported a flare of rheumatoid arthritis during the treatment period, all of which were mild to moderate. The authors indicated that minor gastrointestinal side effects are not uncommon with probiotic therapy, and concluded that short-term consumption of a mixture of probiotics in patients taking sulfasalazine does not appear to result in serious adverse events.

In a randomized, uncontrolled, parallel-arm study, 100 healthy adults consumed lozenges providing either $7.0x10^4$, $1.0x10^6$, $2.0x10^7$, $1.1x10^8$, or $1.5x10^9$ CFU/day of *S. salivarius* K12 for a period of 14 days (Burton *et al.*, 2010). Subjects consumed 1 lozenge per day in the morning after brushing their teeth. Saliva samples were collected at baseline and end of treatment for the quantification of *S. salivarius* K12. At the end of treatment, *S. salivarius* K12 was not detected in the saliva of subjects in the lowest dose group (*i.e.*, $7.0x10^4$ CFU/day). In the other 4 dose groups (*i.e.*, $1.0x10^6$, $2.0x10^7$, $1.1x10^8$, and $1.5x10^9$ CFU/day), the mean counts of *S. salivarius* K12 increased in a dose-dependent manner, ranging from $5.3x10^3$ to $2.6x10^5$ CFU/mL (r=0.9958). The authors reported that the levels of *S. salivarius* K12 colonization were similar to the naturally-occurring levels measured from saliva samples

obtained from a previous study involving 780 children (Burton *et al*., 2010). No adverse events were reported by the subjects.

In the single-arm study conducted by Burton *et al.* (2006a), 14 healthy adults consumed a total of 4 lozenges (1 lozenge every 2 hours for 8 hours), providing $4x10^9$ CFU/day of *S. salivarius* K12 for a period of 3 days. Total counts of *S. salivarius* remained stable through the study, and the microbial composition of saliva remained unchanged. No adverse events were reported by any of the subjects. Similarly, no adverse events were observed in a 40-year-old healthy male who consumed 4 lozenges providing $4x10^{10}$ CFU/day of *S. salivarius* K12 during or after the 3-day treatment period (Horz *et al.*, 2007). *S. salivarius* K12 was detected in the oral cavity for up to 3 weeks, with steadily decreasing counts after day 8 of the study.

In a placebo-controlled, parallel-arm study, 23 healthy adults with halitosis received either 4 lozenges containing 0 (placebo) or $>4x10^9$ CFU/day of *S. salivarius* K12[5] for a period of 3 days (Burton *et al.,* 2006b). Safety-related endpoints and incidences of adverse events were not reported by the study authors.

2.2 Studies Conducted in Children

Di Pierro *et al*. (2014) evaluated the effects of *S. salivarius* K12 consumption in a group of 61 male and female children, 3 to 31 years of age from the Milan area, with a diagnosis of recurrent oral streptococcal disorders. Thirty-one (31) subjects were randomized to daily consumption of *S. salivarius* K12 in slow release tablets providing at least $1x10^9$ CFU per day for 90-days. The remaining 30 subjects served as untreated controls. Tolerability and compliance were high throughout the study. Among the 31 subjects allocated to the *S. salivarius* K12 group tolerability characterized as excellent by 30 subjects. One (1) subject reported tolerance and unacceptable due to the taste of the tablets, and this subject withdrew from the study. No side-effects were reported.

The administration of *S. salivarius* K12 to a group of children with and without recent diagnosis of recurrent oral *streptococcal* pharyngitis/tonsillitis was evaluated by Di Pierro *et al*. (2012). In total 82 children 3 to 12 years of age were recruited to the study including 65 with and 17 without recurrent pharyngitis and/or tonsillitis confirmed by throat swab for group A *Streptococcus*. Forty-five (45) of the 65 children with recurrent phyngitis/tonsillitis were selected for administration of *S. salivarius* K12 tablets, and the remaining 20 were selected for the non-treatment group. The 17 children without a history of recurrent pharyngitis/tonsillitis were selected as an additional non-treatment control group. Subjects in the recurrent-treatment group were instructed to consume a single slow release tablet prior to bedtime after brushing teeth and/or mouthwashing. Tablets were consumed on a daily basis for 90 days and each tablet contained $5x10^9$ CFU of *S. salivarius* K12 at the time of manufacture. Sixteen (16)

[5] A variant of *S. salivarius* K12 resistant to streptomycin was used in this study.

subjects from the recurrent-treated group and 14 subjects from the recurrent non-treated group were enrolled for follow-up lasting an additional 6 months during which time the product was not administered. Safety information was obtained as secondary endpoints and included monitoring of tolerability and side-effects during the 90 days of treatment. The authors reported that the *S. salivarius* K12 tablets were well tolerated and "devoid of side-effects." Four subjects within the treatment group were excluded from study analysis on the basis of poor compliance (missed more than 20 days of treatment), and not due to reported side-effects. Overall, the authors reported that consumption of *S. salivarius* K12 had beneficial effects on oral health. Limitations in the study design include the small sample size and the fact that subjects were not randomized to treatment groups in a placebo controlled double-blind fashion.

In a single-arm study, 19 children who were scheduled to undergo ventilation tube placement surgery were fed a powdered formulation containing freeze-dried *S. salivarius* K12 for 10 days prior to surgery (Power *et al.*, 2008). Approximately 1 g of powdered formula was applied to the subject's tongue surface twice daily, an amount providing an estimated $1x10^{10}$ to $3.4x10^{10}$ CFU/ day of *S. salivarius* K12. Safety-related endpoints and adverse events were not reported by the study authors. *S. salivarius* K12 colonization of the oral and adenoid tissues was reported.

2.3 Studies Conducted In Infants

As part of a multi-center study, Cohen *et al*. (2013) evaluated the consumption of infant formula containing *S. salivarius* K12 in young infants at increased risk of acute otitis media (AOM). Two hundred and twenty-four (224) infants between the ages of 7 to 13 months were enrolled in the study. Inclusion criteria included full-term birth, body weight ≥6 kg, and diagnosed AOM[6] at pre-inclusion visit. In addition, children enrolled in the study were selected on the basis of high risk for AOM, which included children in daycare setting, children with ≥2 siblings and those with at least 1 episode of AOM before recruitment. Infants were randomized to double-blind fashion to 1 of 2 groups fed a formula containing a mixture of probiotics (*i.e.,* $5x10^7$ CFU/g of *S. salivarius* K12, and $2x10^7$ CFU/g each of *S. thermophilus* NCC 2496, and *L. rhamnosus* LPR CGMCC 1.3724 per 100 g formula), and prebiotics (*i.e.,* 1.9 g raftilose/raftiline per 100 g formula) or a placebo formula for a period of 12 months. Powdered formulas were reconstituted in 210 mL of tap water, and guardians were instructed to administer between 300 (minimum) to 630 mL (maximum) of the test formulas to their respective children per day, resulting in a corresponding consumption of $1x10^9$ CFU to $2x10^9$ CFU of *S. salivarius* K12 per infant per day respectively. All participants were treated with antibiotics during the run-in period to ensure that they were free of AOM or that their conditions have improved at baseline. All infants were vaccinated with pneumococcal conjugate vaccine (PCV7). Anthropometric measurements (*i.e.,* weight, length), as well as clinical and otoscopic examinations, were performed every 2 months. Bacterial rhinopharyngeal samples were collected from the first 40 infants of each group at

[6] Acute otitis media was defined as having at least one of the following symptoms: an otorrhea or a tympanic opacity associated with either i) marked congestion; ii) marked bulging; iii) moderated congestion and moderated bulging; iv) fever; v) otalgy or irritability in the ear; or vi) conjunctivitis.

baseline, 2, 4, and 12 months and analyzed for *Streptococcus pneumoniae, Haemophilus influenzae, Branhamella catarrhalis, Streptococcal anginosus,* and *Staphylococcus aureus*. The primary outcome was incidence of AOM. All primary data and safety related measures (adverse events) were analyzed on an intent to treat (ITT) basis.

In total 36 children (18 per group) dropped out before the final visit (V12). Both placebo and treatment formulae were reported to be well-tolerated and no adverse effects on anthropometric measures of growth, a sensitive measure of infant health, were reported. A total of 876 adverse events were reported during the trial, and 93.1% were determined to be not related to treatment (91.6% in treatment group and 94.7% in the control group). One incidence of constipation was considered related to the treatment. After 12 months of treatment no differences in nosopharyngeal flora composition were observed between treatment and controls.

IV.E Additional Safety Considerations Relevant to Live Microorganisms Intended for Use in Food

1. Pathogenicity

1.1 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* were identified in the publicly available literature; however, it should be noted that the majority of cases were related to infection following surgical intervention with poor hygiene control, major tissue trauma, or occurred in immunocompromised individuals. Several cases of iatrogenic infections have been reported following spinal anesthesia (Newton *et al.*, 1994; Berrouschot *et al.,* 1997; Kaiser *et al.,* 1997; Bouhemad *et al.,* 1998; Laurila *et al.*, 1998; Molinier *et al.,* 1998; Yaniv and Potasman, 2000; Idigoras *et al.,* 2001; Trautmann *et al.,* 2002; Conangla *et al.,* 2004; Rubin *et al.*, 2007; CDC, 2010; Wilson *et al.*, 2012; Srinivasan *et al.*, 2012; Suy *et al.*, 2013), myelography (Chitnis *et al.*, 2012), gastrointestinal endoscopy (Carley, 1992; Lee *et al.,* 2000), lumbar/cervical puncture (Torres *et al.,* 1993; Veringa *et al.,* 1995; Cantey and Tamma, 2011), dental manipulation (Bertrand *et al.,* 1997; Romero-Gomez and Larraona, 1999; Maeda *et al.,* 2002), and neurosurgery (Watanakunakorn and Stahl, 1992; Megarbane *et al.,* 2000). Opportunistic infections also have been reported among individuals with suppressed immune systems, including patients with bronchial cancer (Svane, 2000), oral cancer (Sakamoto *et al.,* 1999), colonic cancer (Legier, 1991; Smit *et al.,* 1994; Marinella, 1997; Afek *et al.,* 2004), gastric cancer (Ijyuuin and Umehara, 2012) and neutropenic cancer (Awada *et al.,* 1992; Beighton *et al.,* 1994; Wisplinghoff *et al.,* 1999; Ahmed *et al.,* 2003). Bacteremia caused by *S. salivarius* has been reported in adults (Watanakunakorn and Pantelakis, 1993; Asao *et al.,* 1995; Wisplinghoff *et al.*, 1999; Gautam *et al.*, 2007; Campos Franco *et al.*, 2012), children (Peskine

et al., 1984; West *et al.,* 1998; Paster *et al.,* 2002), and individuals with predisposing medical conditions (Ruoff *et al.,* 1989; Ruiz and Soriano, 1994; Poitrineau *et al.,* 1996). Rare respiratory tract infections (Gentry *et al.,* 1992) and endocarditis (Horaud and Delbos, 1984; Crawford and Russell, 1986) also have been reported to occur. Other rare case reports of infection caused by *S. salivarius* included infection in a patient with a contaminated transplant organ (Heidemann *et al.,* 1989), contamination of amniotic fluid during caesarean section (Berle *et al.,* 1991), acute prostatitis (Suzuki and Horiba, 1991), skin infection (Brook, 1980), tonsillitis (Rajasuo *et al.,* 1996), peritonitis (Redondo Cerezo *et al.,* 2004), and ear infection (Léautez *et al.,* 2002).

Watanakunakorn and Pantelakis (1993) reviewed 203 episodes of *alpha*-hemolytic streptococcal bacteremia over a 10-year period in hospitalized patients. *S. salivarius* accounted for 4 cases of bacterial endocarditis, 15 cases of bacteremia and was involved in 12 cases of polymicrobial bacteremia. Most of the patients were reported to have an underlying condition. Malignant diseases were said to be not uncommon. Wisplinghoff *et al.* (1999) reviewed 57 episodes of bacteremia due to viridians group streptococci in 50 febrile neutropenic patients with hematologic malignancies. Of these *S. salivarius* was associated with just one episode with *Streptococcus mitis* being most often involved. Genomic finger-printing results suggested that most cases were derived from endogenous sources. Beighton *et al*. (1994) also reported on 23 viridians streptococcal isolates from neutropenic cancer patients with *S. salivarius* being identified in 2 of these. Yaniv and Potasman (2000) reviewed iatrogenic meningitis over a 20-year period together with a case report of a patient who had received spinal anesthesia. The literature review revealed 60 cases, with most occurring after spinal anesthesia (n = 27), myelography (n = 20) and diagnostic lumbar puncture (n = 5). The organism was identified in 52 of the cases and 11 of these were *S. salivarius*.

1.2 Presence of Streptococcus Virulence Determinants

The genus *Streptococcus* includes many species, some of which are commensals of the mucosal membranes of the upper respiratory tract, while others (*i.e., S. thermophilus*) are commonly used in the production of fermented dairy products for human consumption. Some species of *Streptococcus* have been identified as human pathogens, and these have been classified according to their group specific polysaccharide (U.S. FDA, 2009). Groups A, B, C and G, and *S. pneumoniae* are the most important pathogens. Group A Streptococci (*Streptococcus pyogenes*) is a human pathogen causing a wide spectrum of diseases, ranging from pharyngitis and impetigo to necrotizing fasciitis and streptococcal toxic shock syndrome (Cunningham, 2000). Group B Streptococci (*Streptococcus agalactiae*) are responsible for many infections in newborns, while Groups C and G *Streptococci* are gaining importance as causing disease in humans, such as toxic shock-like syndrome (Igwe *et al.*, 2003). These pathogenic Streptococci produce a range of virulence factors, and the major identified factors include:

- M protein is a major surface protein of Group A *Streptococci*, which enables the bacteria to resist phagocytosis. It has been suggested that M proteins are also involved in adherence and internalization by epithelial cells (Eyal *et al.*, 2003), and that they may be critical for the development of tissue necrosis (Ashbaugh *et al.*, 1998).

- C5a peptidase is a serine peptidase that specifically inactivates human C5a, one of the principal chemoattractants of phagocytic cells. This enzyme is produced by Group A, B and G *Streptococci* that have been isolated from human disease.

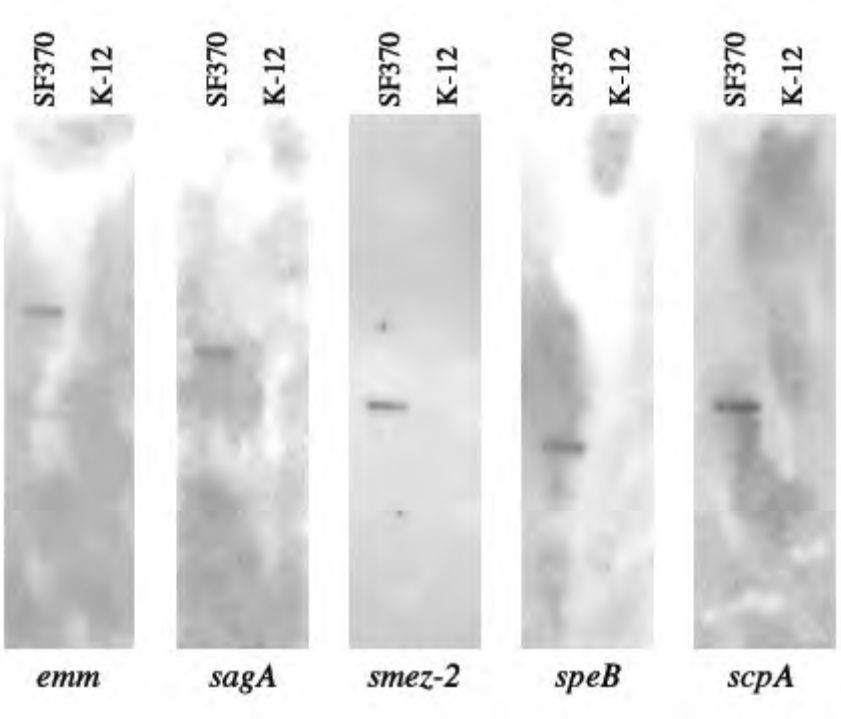
- Streptolysin S (SLS) lyses erythrocytes, polymorphonuclear leucocytes, platelets, and many other cell types. The toxin, which is encoded by the *sagA* gene, is considered to be a contributing factor in the pathogenesis of streptococcal necrotizing soft tissue infection. SLS is produced by virtually all strains of Group A *Streptococci* (Hynes and Tagg, 1986). Group A Streptococci mutants that do not produce SLS were reported to be markedly less virulent than their isogenic parents (Betschel *et al.*, 1998). The *sagA* gene has also been detected in Group C and G Streptococci isolates (Hashikawa *et al.*, 2004).

- Pyrogenic exotoxins are produced by *S. pyogenes*, and cause fever. SPE-B, one of the exotoxins, which is produced by all strains, is a potent cysteine proteinase capable of cleaving many proteins.

- Mitogenic exotoxin (SMEZ-2) is the most potent bacterial super-antigen present in all Group A Streptococci strains (Proft *et al.*, 1999), and is believed to be important in the pathogenesis of invasive streptococcal infections.

Southern blot hybridization and PCR analysis were used to determine whether the chromosomal DNA of *S. salivarius* K12 contained any of the above streptococcal virulence genes using DNA primers for the following virulence genes: *sagA, scpA, smez-2, speB* and *emm*. S. *pyogenes* SF370 (M-serotype 1) was used as a positive control for the virulence genes. Hybridization of any of the virulence primers to *S. salivarius* K12 DNA was not detected using Southern blot analysis, as shown in Figure IV.E-1. Hybridization of the primers to the positive control, *S. pyogenes* strain SF370, was detected, thus confirming the sensitivity of the assay.

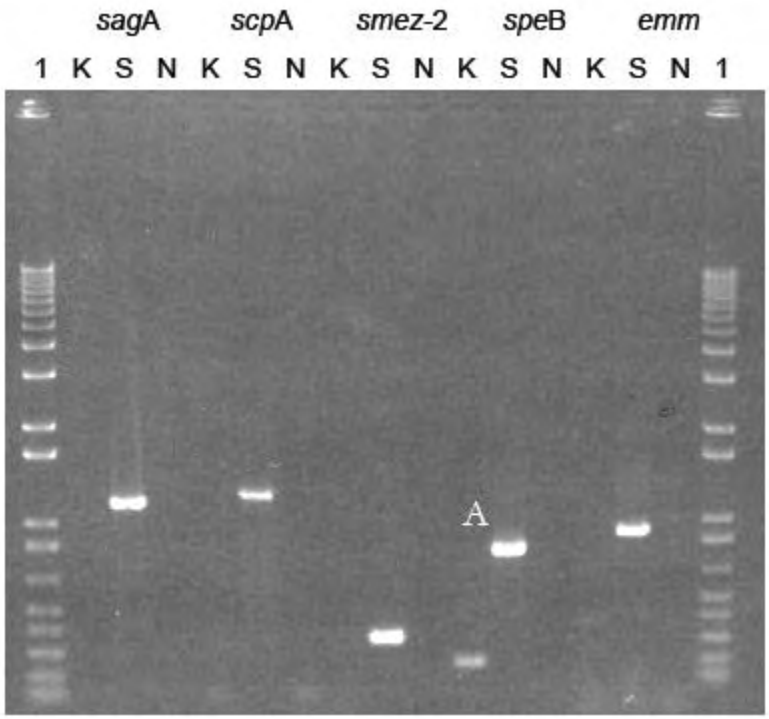
Figure IV.E-1 Southern Blots Hybridized with Streptococcal Virulence Gene Sequences



Lanes containing HindIII-digested DNA from *S. pyogenes* strain SF370 are labeled as SF370. Lanes containing HindIII-digested DNA from *S. salivarius* K12 are labeled as K-12.

As indicated in Figure IV.E-2, no PCR products were detected for *sagA, scpA, smez-2* and *emm* genes; however, a band (labeled A) in the K12 lane of the *speB* PCR product was detected. This band was extracted from the gel and sequenced. The nearest BLAST homology (90% identity) to this sequence was recA from *S. thermophilus*, an unrelated gene.

Figure IV.E-2 PCR Analysis of *Streptococcus salivarius* K12 for the Presence of Streptococcal Virulence Gene Sequences



Lane 1: DNA molecular weight marker (1 kb, Gibco). DNA isolated from *S. salivarius* K12 (K), *S. pyogenes* SF370 (S) and a no DNA control (N).

Overall, the results of the Southern blot hybridization and PCR analyses demonstrate the absence of representative streptococcal virulence genes (*i.e., emm, scpA, speB, smez-2, and sagA*) in *S. salivarius* K12. It should be noted that the list of known or putative virulence factors is considerable and constantly evolving. Virulence is a complex interplay between organism, host and environmental factors. While the presence of key virulence factors has been tested for *S. salivarius* K12, the best indicator of potential virulence is the actual occurrence of infections in use. As discussed above, there is no indication in the literature that *S. salivarius* is associated with virulence. Moreover, the low rate of infection, and lack of significant sequelae following infection suggest that *S. salivarius* is an organism of low virulence potential. Additionally, no cases of infection have been recorded in clinical trials or post-marketing surveillance of *S. salivarius* K12, as discussed in Sections IV.E and IV.G respectively.

1.3 Discussion and Conclusion

Streptococcus salivarius is a normal microorganism of the oropharynx. Colonization of the oropharynx of humans has been reported to occur by 1 month of age (Hegde and Munshi, 1998), and in adults the species 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 between human beings on a daily 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 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 IV.E), 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 have not resulted in adverse effects or changes in biochemical/hematological indicies 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.

2 Antibiotic Resistance

2.1 In Vitro Testing

The antibiotic resistance of *S. salivarius* K12 was evaluated by 2 independent laboratories using the antibiotic disc sensitivity method. Both studies were conducted in accordance to National Committee for Clinical Laboratory Standards (NCCLS) protocols. As shown in Table IV.E-1, *S. salivarius* K12 showed moderate resistance to gentamicin and ofloxacin in both tests.

Table IV.E-1 Antibiotic Resistance of *Streptococcus salivarius* K12

Antibiotic	Concentration (µg)	Zone size (mm)[a]	
		Southern Community Laboratory	Institut fur Mikrookologie
Penicillin	10	30 (S)	32 (S)
Amoxicillin	10	29 (S)	-
Ofloxacin	5	16 (mR)	11 (mR)
Tetracycline	30	26 (S)	30 (S)
Erythromycin	15	27 (S)	-
Gentamicin	10	14 (mR)	10 (mR)
Clindamycin	2	26 (S)	-
Mezlocilin	30	-	35 (S)
Ampicilin	10	-	31 (S)
Cefazolin	30	-	32 (S)
Trimethoprin-	1.25	-	31 (S)
Sulphmethoxazol	23.7	-	-
Piperacilin	30	-	32 (S)
Oxacilin	5	-	30 (S)

mR = moderately resistant; S = sensitive
[a] Assessed by 2 independent laboratories using the Disc Diffusion Method

To determine if the observed antibiotic resistance pattern was genetically stable throughout the history of the organism, and therefore, representative of the antibiotic resistance pattern expected in the master cultures, 3 stock cultures of *S. salivarius* K12 (J89, Lab, and BN21[7]) were further evaluated for their antibiotic resistance patterns. As shown below in Table IV.E-2, there were no differences in antibiotic sensitivity among the *S. salivarius* K12 cultures.

[7] The frozen culture (J89) was obtained from the BLIS Laboratory Culture Collection, Department of Microbiology and Immunology, University of Otago. The laboratory stock culture (Lab) had been sub-cultured on blood agar every 2 weeks for 3 years. The third culture (BN21) was obtained from a commercial batch of *S. salivarius* K12 freeze-dried powder.

Table IV.E-2 Antibiotic Disc Sensitivity of *Streptococcus salivarius* K12 Cultures

Antibiotic	Concentration (µg)	Zone size (mm) of *S. salivarius* Culture		
		J89	Lab	BN21
Penicillin	10	34	34	39
Amoxicillin	10	35	32	35
Ofloxacin	5	18	18	15
Tetracycline	30	28	27	26
Erythromycin	15	30	30	31
Gentamicin	10	15	14	14
Clindamycin	2	29	26	28

To further characterize the antibiotic sensitivity of natural *S. salivarius* isolates in the general population, additional studies were conducted using a number of *S. salivarius* isolates obtained from tongue swabs collected from school children in 1989. Frozen samples from 7 Dunedin school children were selected and spiral plated onto Mitis-salivarius agar containing 3 µg/mL of gentamicin or ofloxacin[8]. All plates were incubated at 37°C, 5% CO_2 in air for 18 to 24 hours. *S. salivarius* colonies which grew on plates containing 3 µg/mL ofloxacin or gentamiicin were selected for further analysis. Two to 4 colonies were selected from each plate, re-grown on blood agar, and their identification checked using API 20 Strep kits. The isolates were then grown in Todd-Hewitt broth at 37°C, 5% CO_2 in air for 18 to 24 hours, then diluted in sterile saline prior to disc sensitivity or agar dilution testing. The antibiotic sensitivity of strains of the natural *S. salivarius* isolates were then evaluated using the disc sensitivity assay. As shown in Table IV.E-3, the pattern of antibiotic sensitivity was similar between all strains tested, and the sensitivity of these isolates to gentamicin and ofloxacin were similar to that observed with *S. salivarius* K12.

Table IV.E-3 Antibiotic Disc Sensitivity of Natural *Streptococcus salivarius* Isolates

Antibiotic	Conc. (µg)	Zone size (mm) *S. salivarius* isolate							
		HD	ToveR	#6	K30	HA	HB	HC	K26R
Penicillin	10	34[a]	29	26	26	26	27	27	28
Amoxicillin	10	35	28	26	29	26	26	28	27
Ofloxacin	5	18	19	17	16	16	18	16	18
Tetracycline	30	28	27	27	22	27	28	28	27
Erythromycin	15	30	28	29	26	28	29	27	27
Gentamicin	10	15	15	16	14	12	12	12	14
Clindamycin	2	29	28	26	26	29	30	28	25

To determine the concentration of antibiotics required for inhibition of growth, a 10^3 sample dilution of the saline samples were spiral plated onto Mueller-Hinton agar plates containing various concentrations of either gentamicin or ofloxacin. The plates were incubated at 37°C,

[8] *S. salivarius* K12 growth was inhibited at this concentration.

5% CO_2 in air for 48 hours. The number of colonies for each antibiotic concentration was determined. Fifty-seven percent (57%) of the subjects had *S. salivarius,* which grew on Mitis-salivarius plates containing ofloxacin, while 86% which grew on gentamicin containing plates (see Table IV.E-4). No growth of *S. salivarius* K12 was observed. For further confirmation of these observations, selected isolates were tested on Mueller-Hinton agar containing the appropriate antibiotic. As shown in Tables IV.E-4 and IV.E-5, 2 subjects (97C84 and 97G61) had isolates that are more resistant to ofloxacin than *S. salivarius* K12, while 2 subjects (97H36 and 97M55) were as sensitive. In addition, 2 subjects (97K41 and 97M55) had isolates more resistant to gentamicin, 1 subject (97M55) had isolates less resistant than *S. salivarius* K12, and 2 subjects (97G61 and 97H36) had a mixture of isolates that were less resistant or as resistant as *S. salivarius* K12.

Table IV.E-4 Growth of *Streptococcus salivarius* on Mitis-salivarius Agar Containing Gentamicin or Ofloxacin

Sample ID	Antibiotic (3 µg/mL)	
	Ofloxacin	Gentamicin
[redacted]	NG	NG
[redacted]	$7.2x10^{4}$ [a]	$1.3x10^{6}$
[redacted]	$1.6x10^{4}$	$3.1x10^{7}$
[redacted]	$7.9x10^{3}$	$2.9x10^{7}$
[redacted]	NG	$2.3x10^{5}$
[redacted]	NG	$2.9x10^{5}$
[redacted]	NG	NG
[redacted]	$1.0x10^{5}$	$8.0x10^{4}$

(4)

NG = no growth
[a] CFU/mL of sample

Table IV.E-5 Growth of *S. salivarius* on Mueller-Hinton Agar Containing Increasing Concentrations of Ofloxacin

Sample ID	Concentration of Ofloxacin (µg/mL)[a]				
	0	0.3	2	3	4
K12	$3.5x10^{4}$ [a]	$2.9x10^{4}$	NG	NG	NG
(b) (4)	$1.7x10^{4}$	$4.4x10^{3}$	$1.1x10^{3}$	NG	NG
	$3.5x10^{4}$	$4.9x10^{3}$	$1.6x10^{3}$	NG	NG
(b) (4)	$3.6x10^{4}$	$3.8x10^{4}$	$1.2x10^{3}$	NG	NG
	$3.9x10^{4}$	$4.9x10^{4}$	$2.3x10^{4}$	NG	NG
(b) (4)	$4.3x10^{4}$	$5.7x10^{4}$	NG	NG	NG
	$7.6x10^{4}$	$7.7x10^{4}$	NG	NG	NG
(b) (4)	$9.3x10^{3}$	$9.1x10^{3}$	NG	NG	NG
	$2.7x10^{4}$	$2.9x10^{4}$	NG	NG	NG

NG = no growth
[a] colony forming units per mL of sample

Although initial observations using the disc sensitivity method suggested that *S. salivarius* K12 may be resistant to ofloxacin and gentamicin, subsequent studies using the agar dilution method indicate that the organism is in fact sensitive to both antibiotics. Complete inhibition of *S. salivarius* K12 growth was observed at concentrations below 2 µg/mL, and the resistance profile of this strain was comparable to or more sensitive to that observed with other natural isolates. These observations are consistent with the literature, and as reported by Doern *et al.* (1996), most species of viridians *Streptococci* are resistant to ofloxacin. Of the 29 *S. salivarius* strains tested by the authors, all were sensitive to ofloxacin as determined by growth inhibition below the 8 µg/mL break-point[9]. Although slight differences in the concentration threshold for gentamicin was noted between dilution studies conducted on Mitis-salivarius agar *vs*. Mueller-Hinton agar[10], concentrations below 8 µg/mL resulted in complete inhibition of *S. salivarius* K12 growth. Although breakpoints for gentamicin resistance have not been set for viridians Streptococci due to the fact that *Streptococcus* spp. and anaerobic bacteria are considered poor targets for gentamicin therapy, a comparison of the antibiotic resistance profile of the natural isolates in Table IV.E-6, an apparent break-point of 10 µg/mL was observed under these experimental conditions. In addition, it should be noted that sensitivity of *S. salivarius* K12 is well below the break-point for the related organism *S. thermophilus*, which has been set by the European Food Safety Authority (EFSA) at a minimum inhibitory concentration (MIC) of 32 mg/L (EFSA, 2012).

Table IV.E-6 Growth of *Streptococcus salivarius* on Mueller-Hinton Agar Containing Increasing Concentrations of Gentamicin

Sample ID	Concentration of Gentamicin (µg/mL)				
	0	3	6	8	10
K12	$4.2x10^{4}$ [a]	$2.5x10^{4}$	$2.4x10^{4}$	NG	NG
(b) (4)	$5.2x10^{4}$	$2.6x10^{4}$	$1.0x10^{3}$	NG	NG
	$3.0x10^{4}$	$2.9x10^{4}$	$5.0x10^{3}$	NG	NG
(b) (4)	$5.5x10^{4}$	$1.0x10^{4}$	NG	NG	NG
	$5.4x10^{4}$	$3.9x10^{4}$	$7.3x10^{2}$	NG	NG
	$4.2x10^{4}$	$1.2x10^{4}$	$3.2x10^{3}$	NG	NG
	$5.1x10^{4}$	$2.3x10^{4}$	$2.3x10^{3}$	NG	NG
(b) (4)	$8.2x10^{3}$	$6.7x10^{3}$	$5.9x10^{2}$	NG	NG
	$1.0x10^{4}$	$9.5x10^{3}$	NG	NG	NG
	$6.1x10^{4}$	$1.4x10^{4}$	$5.5x10^{2}$	NG	NG
(b)	$5.3x10^{4}$	$3.4x10^{4}$	$1.1x10^{4}$	$3.9x10^{1}$	NG
(b) (4)	$3.0x10^{4}$	$3.2x10^{3}$	NG	NG	NG
	$1.0x10^{4}$	$3.3x10^{3}$	NG	NG	NG

[9] Microbiological breakpoints are set by analyzing the Minimum Inhibitory Concentration (MIC) or inhibition zone diameters among several strains within a species. Strains that have acquired bacterial resistance genes will generally clearly deviate from the normal susceptible population and are categorized as resistant.
[10] Interference with the growth medium is known to affect the results of gentamicin MIC analyses (EFSA, 2012).

Table IV.E-6 Growth of *Streptococcus salivarius* on Mueller-Hinton Agar Containing Increasing Concentrations of Gentamicin

Sample ID	Concentration of Gentamicin (µg/mL)				
	0	3	6	8	10
[redacted] (4)	$3.2x10^4$	$2.3x10^4$	$8.7x10^3$	$4.0x10^1$	NG
	$3.8x10^4$	$1.1x10^4$	$6.5x10^3$	NG	NG

NG = no growth
[a] colony forming units per mL of sample

Additional investigations into the antibiotic resistance of *S. salivarius* K12 and 2 other strains of *S. salivarius* (RS1 and ST3) were recently reported by Guglielmetti *et al.* (2010). The experiment was conducted according to conventional broth microdilution protocols using 96-well microtiter plates containing ampicillin, chloramphenicol, erythromycin, oxytetracyline, and vancomycin at concentrations ranging from 1 to 16 µg/mL. Gentamicin sensitivity was evaluated over concentrations of 8 to 84 µg/mL, while vancomycin resistance was tested at concentrations ranging from 16 to 128 µg/mL. Three different liquid media were used: LM17, MRS, and BH1. As shown in Table IV.E-7, *S. salivarius* K12 was reported to be sensitive to ampicillin, chloramphenicol, erythromycin, oxytetracyline, and vancomycin. *S. salivarius* K12 also was reported to be sensitive and resistant to gentamicin depending on the type of medium used; however, the authors noted that interference by the growth media has been reported for gentamicin. Given that *S. salivarius* K12 was not reported to be resistant to gentamicin in earlier experiments, the authors concluded that the effect observed in this experiment was likely an artifact of the growth media. *S. salivarius* K12 also was reported to be resistant to streptomycin in the presence of BH1 media, but not in the presence of LM17 media. In addition, *S. salivarius* K12 was reported to be resistant to kanamycin, and potentially resistant to streptomycin.

Table IV.E-7 Minimum Inhibitory Concentrations of Various Antibiotics against *Streptococcus salivarius* K12, RS1, and ST3[a]

Antibiotic	MIC (µg/mL)[c]								EFSA MIC (µg/mL)[d]
	K12		RS1			ST3			
	BH1	LM17	BH1	LM17	MRS	LM17	BH1	MRS	
Ampicillin	<1	<1	<1	<1	<1	<1	<1	<1	2
Chloramphenicol	<1	<4, >1	<1	<4, >1	<4, >1	<1	<4, >1	<4, >1	4
Erythromycin	<1	<1	<1	<1	<1	<1	<1	<1	2
Gentamicin[b]	<32	**>64**	<16	**<64**	**>64**	<16	<8	**>64**	32
	>16		>8	**>32**		>8			
Kanamycin	**>128**	**>128**	**>128**	**>128**	**>128**	**>128**	>16	**>128**	64
Oxytetracyline	<1	<1	<1	<4, >1	<1	<1	<1	<1	4
Streptomycin	**<128, >64**	<16	**<128, >64**	**<128, >64**	<64, >43	**<128, >64**	<32, >16	**<128, >64**	64
Vancomycin	<1	<4, >1	<1	<4, >1	<4, >1	<4, >1	<4	>1	4

EFSA = European Food Safety Authority; MIC = minimum inhibitory concentration; BH1 = Brain Heart Infusion broth, LM17 = M17 broth supplemented with lactose; MRS = De Man, Rogosa and Sharpe broth
[a] Adapted from Guglielmetti *et al.* (2010).
[b] Possible interference by growth medium reported in literature
[c] MIC's above EFSA limits are in boldface.
[d] Microbiological breakpoints categorizing bacteria as resistant according to EFSA guidelines for the related species *S. thermophilus* (EFSA, 2012).

2.2 Gene Sequence Analysis

Using the available genome sequence of *S. salivarius* K12, *In silico* analyses of the genome was conducted by Nestle using the company's in-house database of antibiotic, virulence and pathogenicity genes, and BlastN software. The search of the *S. salivarius* K12 genome (plasmid and DNA) for the potential existence of 'undesirable genes' was conducted in 2 steps. First, the database was used at the nucleotide level to identify homologous *S. salivarius* K12 genes matching 70% sequence identity and spanning at least 70% of their total length. The database was then searched at the protein level using tBlastN software applying the same coverage thresholds as the first step. To prevent redundancy, only proteins encoded by genes not identified the first step were analyzed. In addition, the identification of each gene meeting the above criteria was further analyzed for the existence of potential transposases by searching the flanking regions of the genes.

Antibiotic resistance can be mediated through the presence of a genetic mutation(s) in a critical protein(s) that is the target of an antibiotic where the mutation prevents interaction between the protein and antibiotic, or antibiotic resistance can be conferred through genes encoding a protein/enzyme that is directly involved in the resistance. The genome annotation identified several nucleotide and protein matches for genes that have been characterized in the literature as conferring antibiotic resistance, including topoisomerase genes targeted by quinolone antibiotics; however, a detailed analysis of the concerned regions within the *S. salivarius* K12 genome revealed that none of the known necessary point mutations was present (Yoshida *et*

al., 1991; Goñi-Urriza *et al.*, 2002; Meier *et al.*, 2003; Norén *et al.*, 2007); therefore, it is not expected that there will be potential resistance to antibiotics such as everninomicin, rifampin and quinolone due to point mutations.

2.2.1 Glycopeptide Antibiotics

Nucleotide matches with 2 putative glycopeptide antibiotics resistance proteins were identified. The best match had 90.34% identity over the total length of the gene. Both genes were located in the publicly available *S. thermophilus* LMD-9 genome. One of the 2 genes encoded for a VanZ-like region, which imparts low-level resistance to the glycopeptide antibiotic teicoplanin; however, *S. salivarius* NCC4010 has been reported to be susceptible to teicoplanin in preliminary *in vitro* tests. The second gene was well-conserved with 84.76% identity over 98% of the *S. thermophilus* gene, but could not be linked to a specific glycopeptide antibiotic.

2.2.2 beta-Lactam Antibiotics

The *beta*-lactamase is a type of enzyme (EC 3.5.2.6) produced by some bacteria responsible for resistance to antibiotics containing *beta*-lactam rings, which directly inactivate these antibiotics by cleaving the ring structures within these types of molecules. Common *beta*-lactam containing antibiotics include penicillins, cephalosporins, cephamycins and carbapenems. As shown in Table IV.E-8, 4 nucleotide matches with 88.77 to 93.66% identity were identified for different genes encoding *beta*-lactamase or metallo-*beta*-lactamase enzymes. In addition, a match at the protein level with a metallo-*beta*-lactamase protein (NCBI ID number = AAM99608.1; 82.09% identity and 100% coverage) was identified, as well as a "*beta*-lactam resistance factor" present in *S. agalactiae* 515 (NCBI ID number=EAO70085.1, 74.07% identity and 82% coverage). However, *S. salivarius* has been shown to be susceptible to antibiotics from different *beta*-lactam classes (*i.e.,* penicillins, cephalosporins, and carbapenems) (see Section VIII.E Post market Surveillance).

Table IV.E-8 *beta*-Lactamases and Metallo-*beta*-Lactamases Present at the Nucleotide Level

Antibiotic	NCBI ID Number	% Identity	% Coverage
beta-Lactamase	ABJ65406.1	91.57	100
	ABJ66775.1	88.77	99
Metallo-*beta*-lactamase	AAV60823.1	88.93	100
	AAV60860.1	93.66	100

2.2.3 Azaleucine

Azaleucine is a naturally-occurring amino acid antibiotic that can exert its antibacterial action by substituting for leucine during protein synthesis resulting in a dysfunctional protein product. Azaleucine resistance is due to the combined absence of *azlB* gene and overexpression of *azlC*

and *azlD* genes. Upon analysis, a close match to a protein from *S. thermophilus* LMD-9 entitled "Predicted branched-chain amino acid permease (azaleucine resistance)" (NCBI ID number=ABJ66885.1, 89.91% identity and 100% coverage) was identified. This protein is encoded by *azlC* gene. No matches to either the *azlB* gene, or the *azlD* gene (necessary to confer resistance) were found. It was concluded that resistance to azaleucine would not be expected.

2.2.4 Aminoglycosides

Several good matches were found at the nucleotide level with *Streptococcus oralis*, *S. pneumoniae*, and *S. mitis* genes encoding for an 'aminoglycoside adenylyltransferase'. There was 89% identity over the first and third part of the gene, and at the protein level (tBlastN) the best match was with *S. pneumoniae*, covering 93% of the protein length with 78% identity. Based on the *in vitro* observations of resistance to the aminoglycoside antibiotics, streptomycin and kanamycin, it is likely that *S. salivarius* K12 contains an aminoglycoside resistance gene. No transposase was identified in the flanking regions of any the gene.

2.2.5 Macrolides

At the nucleotide level, 2 very close matches were found at 2 different loci in the genome for genes involved in macrolide antibiotics resistance. One gene encoded for a protein annotated as resistance to 14- and 15-membered macrolides in *S. agalactiae* (V. Perreten ID=U92073, 67% identity and 96.79% coverage). The other gene encoded for a protein annotated as "macrolide efflux protein" in *S. thermophilus* CNRZ 1066 (NCBI ID number=AAV63103.1, 97.79% identity and 100% coverage). As reported by Guglielmetti *et al.* (2010), *S. salivarius* K12 is sensitive to erythromycin (a 14-membered macrolide); no 15-membered macrolide antibiotic has been tested.

2.2.6 Bacitracin

In infants, bacitracin is sometimes administered intramuscularly for the treatment of pneumonias. The *mrb* gene cluster (*mrbA* to *mrbD*) involved in bacitracin resistance was found. Specifically, the *mrbA* gene encoding for an apparent 'ABC-type bacitracin resistance protein A' was found at the protein level with 85.71% identity, covering 100% of the same gene in *S. sanguinis* SK36 (NCBI ID number=ABN45065.1). An additional protein match was found with an 'uncharacterized bacitracin resistance protein' from *Streptococcus suis* O5ZYH33 (NCBI ID number=ABP90845, 74.73% identity and 100% coverage). No transposase was identified in the flanking regions of the genes.

2.2.7 Other Virulence and Toxin Encoding Genes

A good protein match was found with an “Adherence and virulence protein A” from *Streptococcus agalactiae* COH1 (protein ID=EAO76664.71, 79.35% identity and 100% coverage). A good match also was found with a ‘Pneumococcal surface adhesion A’ from *S. anginosus* (protein ID = Q9L5X1, 85% identity and 92% coverage). The functional importance of these adhesion genes is not known.

Overall, based on the results of *in vitro* and bioinformatic analyses, it can be concluded that *S. salivarius* K12 contains an ‘acquired’ kanamycin resistance determinant. A suspected genetic determinant potentially conferring resistance to bacitracin also was suggested by bioinformatic analyses. However, the results of the bioinformatic analysis also demonstrated that these genes were not associated with transposable elements and therefore are of low risk for potential transference to other organisms.

3. Additional Metabolic Properties

3.1 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. The production of bacteriocins is ubiquitous among gram positive bacteria, and almost all bacteriocins have a net negative charge, and often contain regions that are hydrophobic and/or amphiphilic; physicochemical properties that are critical to their bactericidal/bacteriostatic activities (Eijsink *et al.*, 2002). Although a standardized naming system for bacteriocins is yet to be universally recognized, these compounds are generally accepted to be categorized into 3 classes (Class I through III), which are further divided into several subtypes as described in Table IV.E-9. A newly defined forth class of bacteriocins constitutes cyclic bacteriocins, ribosomally-synthesized peptides which are post-translationally processed such that the first and last amino acids of the mature peptide are covalently bonded (Heng *et al*., 2007).

Table IV.E-9 Bacteriocin Classification[a]

Class	Subtype	Features	Examples
I - Lantibiotics	A	Linear, amphipathic, small cationic peptides Further divided into subtype A-I and A-II based on their size, charge, and leader peptide sequence	Subtype AI: Nisin, streptin Subtype AII: Salivaricin A,B, lacticin 481
	B	Globular anionic or neutral peptides	Mersacidin, cinnamycin
II - Non-modified heat-stable bacteriocins	IIa	Small (<10 kDa) heat-stable pediocin-like peptides, active against Listeria	Pediocin PA-1, sakacins A and P, luecocin A.
	IIb	Multi-component bacteriocins	Lactococcins G and F, Lactacin A, B and F
III - Large heat-labile bacteriocins	IIIa	Large (>10 kDa) molecules sensitive to heat	Lysostaphin
	IIIb		

[a] Adapted from Heng *et al.* (2007) and Eijsink *et al.* (2002)

Lantibiotics, which are antimicrobial peptides produced by closely-related gram positive bacteria (Upton *et al.*, 2001), are among the most well-studied group of bacteriocins. One of the most well-known lantibiotics is nisin, 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 000065)[11].

Like most bacteriocins, Type A lantibiotics 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 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. However, given the differences in the mechanisms of action between antibiotics and lantibiotic bacteriocins (*i.e.*, nisin and salivaricin), 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.

[11] Available from the website: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=65 (U.S. FDA, 2001)

S. salivarius K12 produces 2 Class IIb peptide lantibiotic bacteriocins: salivaricin A2 (SalA2) and salivaricin B (SboB) (Wescombe *et al.*, 2006). Salivaricin lantibiotics are synthesized ribosomally and undergo several post-translational modifications including amino acid dehydration and thioether bridge formation between side chains of cysteine and serine or threonine (Upton *et al.*, 2001; Eijsink *et al.*, 2002). Using PCR analysis to detect bacteriocin producing *S. salivarius* isolates, Dierksen *et al.* (2007) identified *salA*, the gene encoding SalA2, in 11 out of 18 strains. The production of SalA2, SboB, and a number of other bacteriocins were identified in 9 of 28 *S. salivarius* strains (Wescombe *et al.*, 2006); an observation that was in all cases associated with the presence of a megaplasmid. Localization of the bacteriocins to these megaplasmids was confirmed by the absence of bacteriocin-like inhibitory substances activity in plasmid-free *S. salivarius* K12 (*S. salivarius* K12P) cultures. Further genomic investigations of *S. salivarius* K12 verified the localization genes encoding these BLIS lantibiotics (*salA2* and *spoB*) to a 190 kb megaplasmid. The authors also reported that the megaplasmid could be transferred *in vivo* from *S. salivarius* K12 strains to the plasmid-free *S. salivarius* K12P strains following co-inoculation of both organisms into the oral cavity of 3 volunteers; however, whether *S. salivarius* K12 can transfer these bacteriocin loci to other *S. salivarius* strains or to different streptoccocal species is unknown.

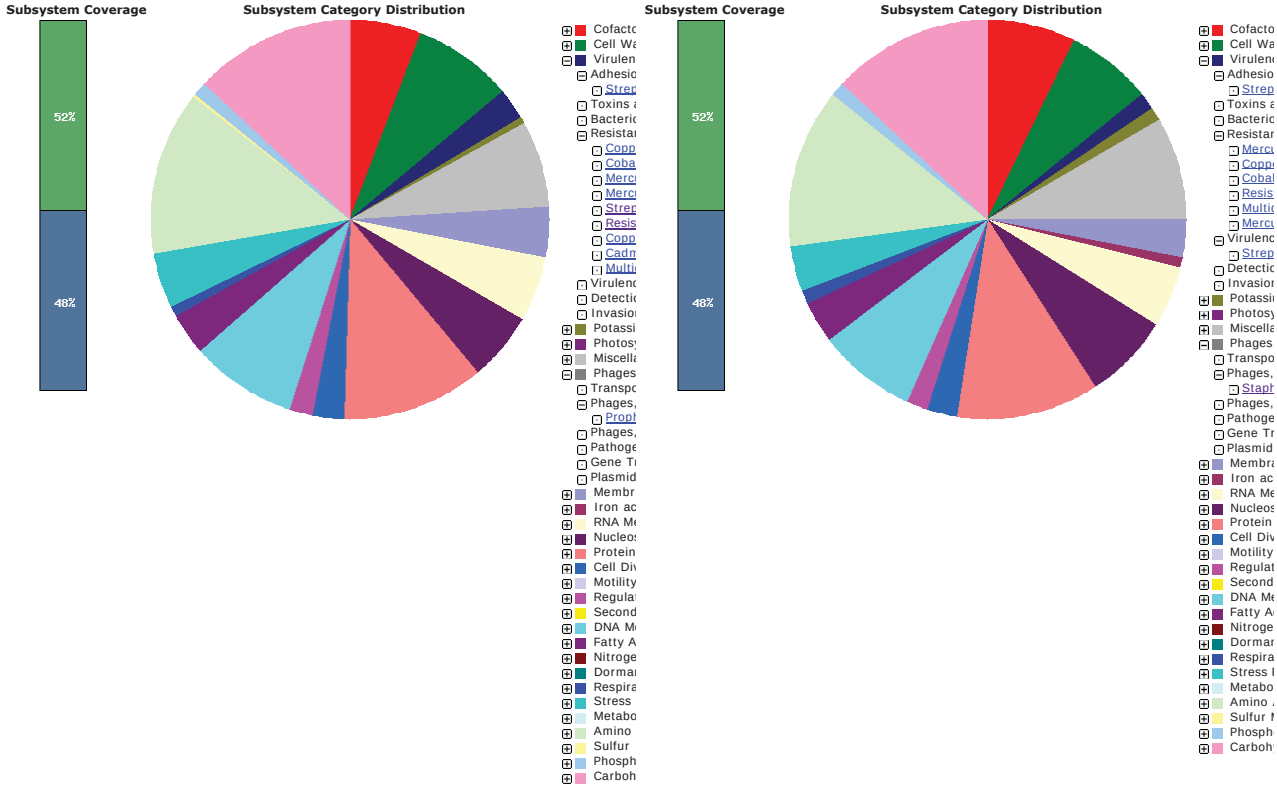
As mentioned above, the lantibiotics present in *S. salivarius* K12 (*i.e.,* SalA2 and SboB) are located on a large plasmid (Wescombe *et al.*, 2006; Hyink *et al.*, 2007). Since these lantibiotics function *via* surfactant-like effects and disrupting the bacteria cell membrane of select microorganisms, the mechanisms of action of SalA2 and SboB do not present safety concerns with respect to the potential of spreading antibiotic resistance to clinically-significant antibiotics to pathogenic organisms. More importantly, both lantibiotics have been reported in several *S. salivarius* strains (Hyink *et al.*, 2007), which suggests that such lantibiotic-producing organisms are present in a number of human commensals, further supporting the safety of these compounds with respect to the proposed use of *S. salivarius* K12 as an ingredient in foods.

IV.F Bioinformatic Comparison to *Streptococcus thermophilus*

Based on the close genetic relation between *S. salivarius* and *S. thermophilus*, and the long-history of safety use of *S. thermophilus* in food production, a bioinformatic comparison of the *S. salivarius* K12 genome with a publically available genome sequence for *S. thermophilus* CNRZ1066 was conducted. A strong conservation of biological sub-systems between the strains would further corroborate the safety of *S. salivarius* K12. An analysis of the DNA sequence and annotation of *S. salivarius* K12 was conducted using Rapid Annotation using Subsystem Technology (RAST). A subsystem is a set of functional roles that together implement a specific biological process or structural complex. Subsystems represent the collection of functional roles that make up a metabolic pathway, a complex (*e.g.,* the ribosome), or a class of proteins. Figure IV.F-1 is a graphical comparison of the subsystem of *S. salivarius*

K12 to *S. thermophilus* CNRZ1066 based on DNA sequence. The subsystem architecture was highly homologous between the two genome sequences.

Figure IV.F-1 Genome Subset Comparison of *Streptococcus salivarius* K12 and *Streptococcus thermophilus* CNRZ1066



Further characterization of the virulence subsystem, contain subcategories relating to adhesion, resistance to antibiotics, and other toxins is presented in Table IV.F-1. The 2 organisms were observed to share some putative genes within the virulence subsystem categories. These genes were largely related to resistance and adhesion traits and no genes encoding Streptococcal toxins or other recognized virulence determinants of importance to Streptococcal pathogens (*e.g.*, M protein, C5a peptidase, streptolysin S, pyrogenic extoxins, mitogenic exotoxin) were not identified. Although genes related to vancomycin and fluoroquinoline tolerance were identified in *S. salivarius* K12 genome, *in vitro* studies demonstrated that *S. salivarius* K12 is sensitive to vancomycin. Sensitivity to the fluoroquinolone ofloxacin was also demonstrated. Analyses of protein sequences and BLAST dot plots also indicate close identity between *S. salivarius* K12 and *S. thermophilus* CNRZ1066, which contrast markedly to the large differences between *S. salivarius* K12 and the pathogen *S. pyogenes* M1 GAS.

Table IV.F-1 Virulence, Disease and Defense Subsystem in *Streptococcus salivarius* K12 (A) or *Streptococcus thermophilus* (B)

Subsystem	Role	A	B
Adhesion			
Streptococcus pyogenes recombinatorial zone	Chaperonin (heat shock protein 33)	yes	yes
Bacteriocins, ribosomally synthesized antibacterial peptides			
Colicin V and Bacteriocin Production Cluster	Colicin V production protein	yes	no
Regulation of Virulence			
VraSR Two-Component Regulatory System	Sensor histidine kinase VraS	yes	yes
VraSR Two-Component Regulatory System	Transporter associated with VraSR	yes	yes
VraSR Two-Component Regulatory System	Two component transcriptional regulator VraR	yes	yes
Resistance to antibiotics and toxic compounds			
Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein	yes	yes
Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein CzcD	yes	yes
Cobalt-zinc-cadmium resistance	Hydroxyacylglutathione hydrolase (EC 3.1.2.6)	yes	yes
Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family	yes	yes
Copper homeostasis	Copper chaperone	yes	yes
Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)	yes	yes
Copper homeostasis	Negative transcriptional regulator-copper transport operon	yes	yes
Mercuric reductase	Mercuric ion reductase (EC 1.16.1.1)	yes	yes
Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase	yes	yes
Mercury resistance operon	Mercuric ion reductase (EC 1.16.1.1)	yes	yes
Multidrug Resistance Efflux Pumps	Multidrug resistance efflux pump PmrA	yes	yes
Resistance to fluoroquinolones	DNA gyrase subunit A (EC 5.99.1.3)	yes	yes
Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)	yes	yes
Resistance to fluoroquinolones	Topoisomerase IV subunit A (EC 5.99.1.-)	yes	yes
Resistance to fluoroquinolones	Topoisomerase IV subunit B (EC 5.99.1.-)	yes	yes
Cadmium resistance	Cadmium efflux system accessory protein	yes	no
Cadmium resistance	Cadmium resistance protein	yes	no
Cobalt-zinc-cadmium resistance	Permease of the drug/metabolite transporter (DMT) superfamily	yes	no
Cobalt-zinc-cadmium resistance	Ribosyl nicotinamide transporter, PnuC-like	yes	no
Copper homeostasis: copper tolerance	Cytoplasmic copper homeostasis protein cutC	yes	no
Streptococcus pneumoniae Vancomycin Tolerance Locus	ABC transporter membrane-spanning permease, Pep export, Vex1	yes	no
Streptococcus pneumoniae Vancomycin Tolerance Locus	ABC transporter membrane-spanning permease, Pep export, Vex3	yes	no
Streptococcus pneumoniae Vancomycin Tolerance Locus	ABC transporter, ATP-binding protein Vex2	yes	no
Streptococcus pneumoniae Vancomycin Tolerance Locus	Sensor histidine kinase VncS	yes	no

Table IV.F-1 Virulence, Disease and Defense Subsystem in *Streptococcus salivarius* K12 (A) or *Streptococcus thermophilus* (B)

Subsystem	Role	A	B
Streptococcus pneumoniae Vancomycin Tolerance Locus	Two-component response regulator VncR	yes	no
Other			
Streptococcus pyogenes Virulome	Plasmin(ogen) receptor	no	yes

Figure IV.F-2 Bioinformatic Comparison of the *Streptococcus salivarius* K12 Genome with Streptococcal Genomes of Dairy or Pathogenic origin

***S. salivarius* K12/ *S. thermophilus* CNRZ1066** ***S. salivarius* K12/ *S. pyogenes* M1 GAS**

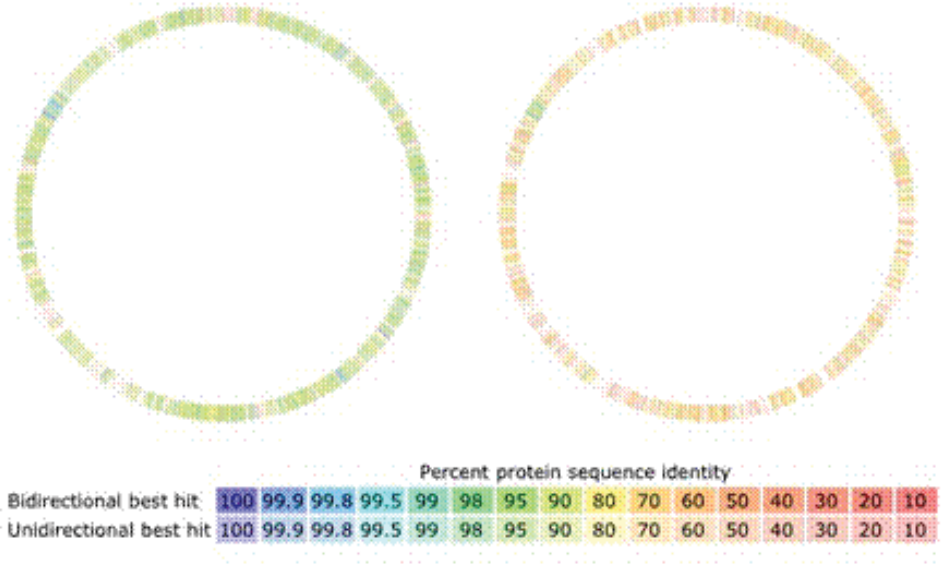
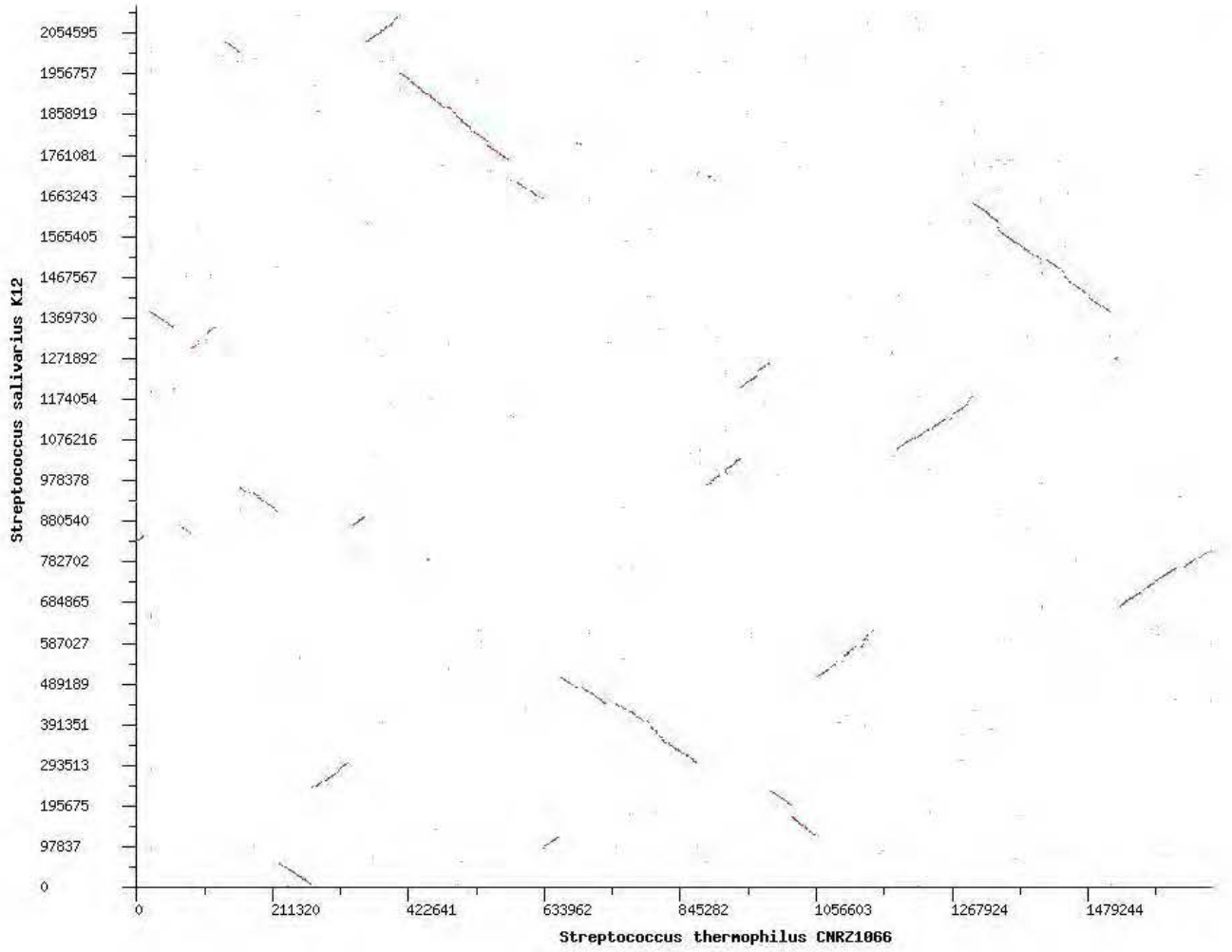
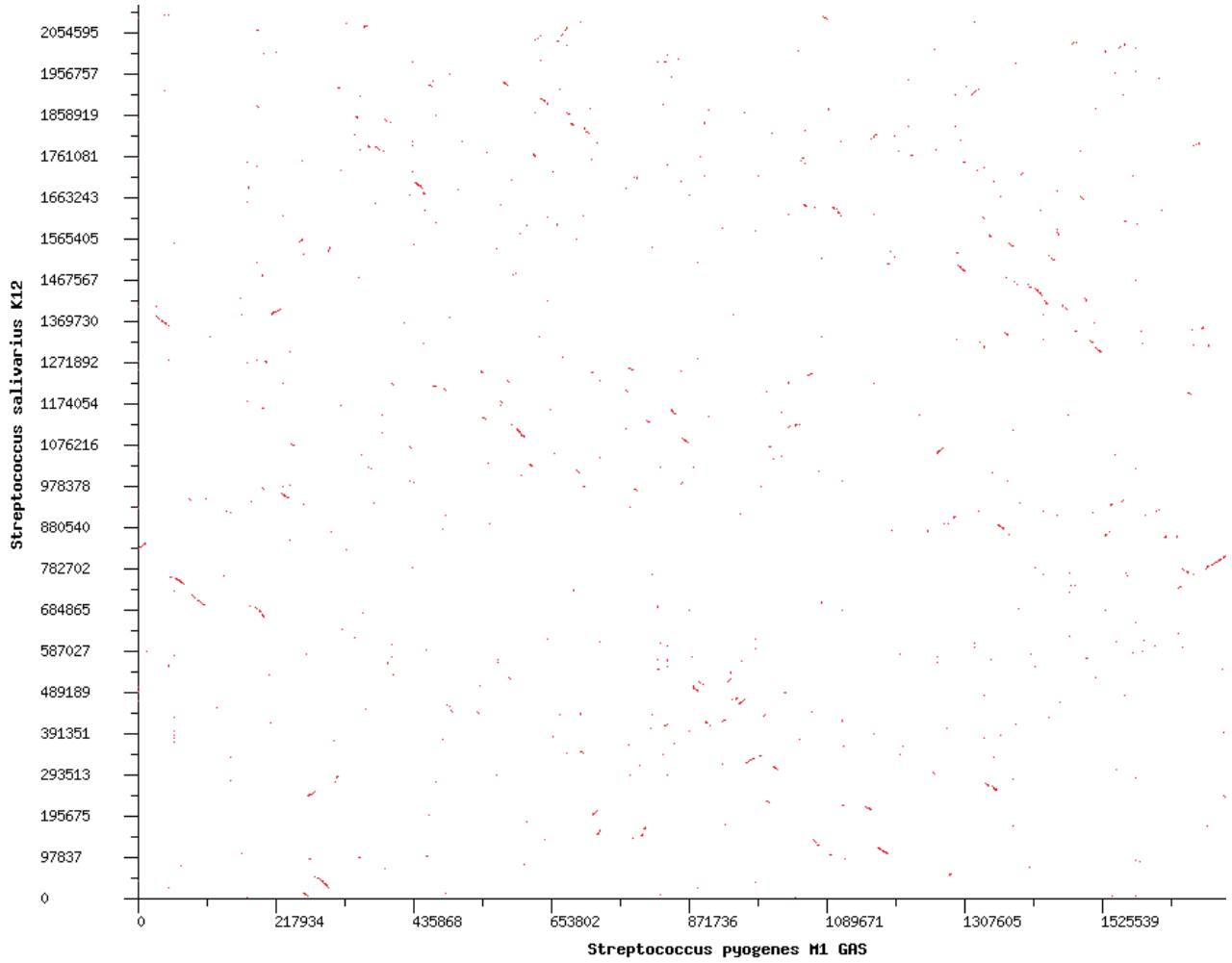


Figure IV.F-3 Blast Dot Plots of *Streptococcus salivarius* K12 Genome Compared to Streptococcal genomes of dairy or pathogenic origin

A. *S. salivarius* K12 *vs. S. thermophilus* CNRZ1066



B. *S. salivarius* K12 *vs. S. pyogenes* M1 GAS



IV.G Post-Market Surveillance

Supplement products (*i.e.,* lozenges) containing *S. salivarius* K12 are currently marketed in New Zealand. From May 2002 to June 2011, in excess of 3 million doses of *S. salivarius* K12 have been sold in New Zealand. During this period, 12 adverse events have been reported by individuals consuming supplement products containing *S. salivarius* K12. These reports are discussed below and summarized in Table IV.G-1.

Two adults complained they felt sick after taking the lozenges, but follow up of the complaints resulted in them not elaborating further on the complaint. One adult went to their dentist with an infected gum after using an *S. salivarius* K12 product. A swab from the site of infection showed the infection was due to *beta*-hemolytic Staphylococci, and not due to *S. salivarius* K12. One adult suffered a worsening of a pre-existing sore throat, but the symptoms completely resolved

after receiving a course of antibiotics from their general practitioner. Another adult complained of dry throat and diarrhea after starting to take a *S. salivarius* K12 tablet. Testing of a saliva sample showed neither presence of *beta* hemolytic streptococci nor any detectable colonization by *S. salivarius* K12. None of the adverse event reports were considered to be possibly or probably related to the consumption of *S. salivarius* K12.

Table IV.G-1 Summary of Adverse Event Reports in Individuals Consuming *Streptococcus salivarius* K12 in New Zealand

Report Reference and Date	Patient Details (age, sex)	Other Medicines	Adverse Reaction	Comments	Causality
ARF-1 9-5-02	Adult, F	Unknown	Cold got worse	n/a	Unlikely
ARF-2 26-6-02	Adult (sex NR)	Unknown	Severe headaches	Not lactose intolerant	Unlikely
ARF-3 1-7-02	Adult (sex NR)	Unknown	Stiff, swollen arm	Previous cancer	Unlikely
ARF-4 22-7-02	Adult, M	Unknown	Sore throat and abscess	Abscess, no *S. salivarius* isolated	Unlikely
ARF-5 9-7-03	Age NR, Male	Unknown	Swelling on jaw	No history of allergies	Unlikely
ARF-6 10-7.03	Adult, M	Unknown	Fever and diarrhea	n/a	Unlikely
ARF-7 27-5-04	Adult, F	No	Cough and dry throat	No allergies	Unlikely
ARF-8 7-7-04	Adult, F	No	Sore throat, diarrhea	May be reaction to chlorhexidine	Unlikely
ARF-9 13-10-04	Adult, M	No	Sore throat	No contamination of lozenges	Unlikely
ARF-10 15-11-04	Adult, F	No	Gastrointestinal upset	Symptoms stopped on taking	Unlikely
ARF-13 23-12-06	Adult, M	No	Gastrointestinal upset	n/a	Unlikely
ARF-15 1-6-07	Adult, F	No	Sore throat	Used with chlorhexidine	Unlikely

F = female; M = male; n/a = not available; NR = not reported

Some of the New Zealand manufactured finished material also has been sold in Australia (in excess of 2.5 million doses). There have been at least 4 reported adverse events in the Australian market since 2005, which included a swollen face (2005), a genital rash on the partner of a *S. salivarius* K12 user (2007), and 2 gastrointestinal upsets (2011). *S. salivarius* K12 consumption was considered to be an unlikely cause of these adverse events since it was often consumed in combination with other products, such as antimicrobial mouthwash.

S. salivarius K12 has been sold as an ingredient to third party manufacturers in the U.S. for dietary supplement products with more than 50 million raw doses sold. There has been at least 1 adverse reaction complaint against a third party product (BioGuard, Imaginetix) containing

S. salivarius K12 that has been reported to BLIS Technologies. This person complained of stomach cramping, diarrhea and other symptoms, but clinical and laboratory investigations, combined with patient's previous medical history, did not indicate *S. salivarius* K12 infection.

IV.H Expert Panel Evaluation

BLIS Technologies has determined that freeze dried *Streptococcus salivarius* K12 powder, as described herein, is GRAS for food and beverage products as described in Table I.D-1, on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of freeze dried *S. salivarius* K12, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of freeze dried *S. salivarius* K12 powder as a component of food. The Expert Panel consisted of the following qualified scientific experts: Dr. Michael Pariza, Ph.D. (University of Wisconsin), Dr. John Doull, Ph.D., M.D. (The University of Kansas Medical Center), and Dr. Ronald Kleinman, M.D. (Massachusetts General Hospital, Harvard Medical School).

The Expert Panel, convened by the BLIS, independently and critically evaluated all data and information presented herein, and also concluded that freeze dried *Streptococcus salivarius* K12 powder was GRAS for food and beverage uses as described in Table I.D-1., based on scientific procedures. 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 *Streptococcus salivarius* K12 powder is presented in Appendix A.

IV.I Conclusion

Based on the above data and information presented herein, BLIS has concluded that the intended food-uses of the *Streptococcus salivarius* K12 ingredient, as described in Section I.D, are GRAS based on scientific procedures. General recognition of BLIS's GRAS self-affirmation is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of the freeze-dried *S. salivarius* K12 powder in food, who similarly concluded that the intended uses of the *S. salivarius* K12 ingredient described herein are GRAS.

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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
168—Sweeteners and table sirups	168.111	Dextrose monohydrate
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS).
172—Food additives permitted for direct addition to food for human consumption	172.840	Polysorbate 80
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
	184.1150	Bacterially-derived protease enzyme preparation
	184.1444	Maltodextrin
	184.1736	Sodium bicarbonate
	184.1763	Sodium hydroxide
	184.1983	Bakers yeast extract

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Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Use of *Streptococcus salivarius* K12 in Food

April 02, 2015

Introduction

Blis Technologies Ltd. (BLIS) intends to market *Streptococcus salivarius* K12 (*S. salivarius* K12) 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 (baby, infant, and toddler foods; 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; soft sugar-free candies; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices) at use-levels of up to 20 mg/serving (providing up to $2.0x10^9$ CFU/serving).

BLIS convened a panel ("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* K12 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. Michael Pariza, Ph.D. (University of Wisconsin), Dr. John Doull, Ph.D., M.D. (The University of Kansas Medical Center), and Dr. Ronald Kleinman, M.D. (Massachusetts General Hospital, Harvard Medical School).

The Panel, independently and collectively, critically evaluated a dossier which included a summary of the scientific information on *S. salivarius K12* 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* K12 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 Panel evaluated other information deemed appropriate or necessary.

Following its independent, critical evaluation of such data and information, the Panel convened on Thursday July 21, 2011, and unanimously concluded that *S. salivarius K12*, meeting appropriate food-grade specifications as described in the supporting dossier [**Documentation Supporting the Evaluation of *Streptococcus salivarius* K12 as Generally Recognized as Safe (GRAS) for Use in Foods**], and manufactured according to current Good Manufacturing

Practice (cGMP), is GRAS under the conditions of intended use described in Table A-1. This GRAS determination was based on scientific procedures, and a summary of the basis for the Expert Panel's conclusion is provided below.

Identity and Manufacturing

The ingredient that is the subject of this GRAS evaluation is *Streptococcus salivarius* K12 freeze dried powder. *S. salivarius* K12 is a Gram-positive, non-hemolytic, non-spore forming cocci. The organism can be selectively cultivated on Mitis salivarius agar, and is further characterized phenotypically based on its unique antimicrobial spectrum (P-type 777) through the use of a deferred antagonism test against 9 bacterial indicator strains. 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 and has been deposited at DDBJ/EMBL/GenBank under accession number ALIF00000000 (Barretto *et al*., 2012). Analysis of the entire 16S rRNA gene sequence using a database of DNA sequences held at the NCBI, National Institutes of Health by using BLAST N programme, identified matches to *S. salivarius* and *Streptococcus thermophilus.* The combination of 16S sequence data, and information provided by phenotypic profiling were sufficient to confirm the species identity as *S. salivarius*. Strain K12 was shown to display a unique DNA fingerprint when analyzed by ERIC-PCR molecular typing, or 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.

Streptococcus salivarius K12 is currently deposited in the American Type Culture Collection (ATCC) as ATCC BAA-1024, the German Collection of Microorganisms and Cell Cultures (DSM 13084) and the Microbial Fermentation Unit at Fonterra, New Zealand. Master cultures and working cultures are maintained at BLIS Technologies Ltd., and the Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand. The Panel reviewed information provided by BLIS describing the chemistry and manufacturing of the *S. salivarius* K12 ingredient. The Panel reviewed documentation supporting that *S. salivarius* K12 is fermented under cGMP using permitted and suitable fermentation processing-aids. The Panel 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 of these materials are currently permitted for use in food in the U.S. Suitable food-grade specifications have been developed for freeze-dried *S. salivarius* K12 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 Panel.

Intended Uses and Consumption Estimates

Consumption estimates for the intake of *S. salivarius* K12 following introduction to the United States 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 NHANES (CDC, 2006, 2009; USDA, 2009), which provides the most appropriate data for evaluating food-use and food consumption patterns in the U.S. Among users only, the mean intake of *S. salivarius* K12 by the total U.S. population from all proposed food-uses was estimated to be $9.8x10^9$ CFU/person/day or $2.0x10^8$ CFU/kg body weight/day. On an individual population basis, the greatest mean all-user intake of *S. salivarius* K12 on an absolute basis was determined to occur in male teenagers at $1.2x10^{10}$ CFU/person/day. Foods containing *S. salivarius* K12 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* K12 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.

Information Establishing Basis for Safety

History of Safe Consumption

The Panel reviewed a large body of safety data during their assessment of the safety of *S. salivarius* K12; this information included both strain-specific information and relevant safety data on non-related strains of *S. salivarius*. The Panel noted that strains of *S. salivarius* have a history of use as a starter culture for the manufacture of cheese and yogurt. Strains of *S. salivarius* also have been demonstrated to be natural residents of the human oral cavity, and have been identified in infants within 2 days after birth. Furthermore, *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).

Persistence and Metabolic Fate

It is well established that the indigenous microflora population of most animals are intrinsically highly stable and resistant to colonization by exogenous microorganisms, and permanent lifelong colonization by ingested microorganisms is rare (WHO/FAO, 2009). Following acute consumption, or discontinuation of repeated consumption of *S. salivarius* K12, the presence of *S. salivarius* strains displaying a K12-like phenotype within saliva samples typically return to baseline levels within 14 days (Burton *et al*., 2006a; Horz *et al.*, 2007; Burton *et al*., 2011). The colonization efficiency of *S. salivarius* K12 displays significant inter-individual variability, and is influenced by the local microflora population and corresponding availability of colonization sites

within the oral cavity (Burton *et al.*, 2006b; Power *et al.*, 2008). Increased persistence of *S. salivarius* K12 within the oral cavity can occur under conditions in which antibiotics and/or oral antibacterial mouthwashes are used prior to consumption of *S. salivarius* K12 (Burton *et al*., 2006b). The effect of *S. salivarius* K12 colonization on the indigenous microflora has been investigated, and even under conditions conducive to optimal *S. salivarius* K12 colonization (pre-rinse with antibacterial mouthwash), no overt changes in the overall microbial composition relative to that characterized at baseline prior to consumption of K12 was reported (Burton *et al*., 2006b).

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 by gavage with a microorganism mixture containing *L. acidophilus* LA741 ($3.9x10^9$ CFU), *L. 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, 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 mucus 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." Although similar studies evaluating the viability and colonization capacity of the organism in humans are not available, *S. salivarius* K12 cannot survive prolonged exposure to acidic environments (Chen *et al.*, 2000); other phenotypes that are characteristic of gut commensals adapted to survive within the gastrointestinal environment (bile salt hydrolase activity and resistance to digestive enzymes) have not been detected within the genome of *S. salivarius* K12. Although viable *S. salivarius* isolates have been cultured from infant and adult fecal samples, studies of human intestinal microbiota indicate the *Streptococcal* species constitute a minor fraction of the gut microflora (Chen *et al.*, 2000; Benson *et al.*, 2010; Arumugam *et al.*, 2011); active survival and growth of *S. salivarius* K12 within the gastrointestinal tract is unlikely. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the cellular constituents would be represented as innocuous compounds (proteins, lipids, carbohydrates) that would be metabolized by well established pathways. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the feces.

Animal Studies

A review of data from published animal studies and the original study reports evaluating the toxicity of *S. salivarius* K12 as well as published studies on non-related strains did not identify toxicity or pathogenicity concerns. The acute and sub-acute toxicity of *S. salivarius* K12 freeze-dried powder was evaluated by Burton *et al*. (2010). In the acute toxicity study, no adverse

effects were observed in groups of male and female Sprague-Dawley rats (250 to 300 g) administered *S. salivarius* K12 freeze-dried powder as single gavage solutions at doses of up to $8x10^{10}$ CFU/rat (Burton *et al*., 2010). In the 28-day dosed-feed study, groups of 20 male and female Sprague-Dawley rats (350 to 500 g) were randomized to 1 of 4 groups administered the following treatments: 1) 7.5 mg/kg body weight *S. salivarius* K12 freeze dried powder ($1.25x10^8$ CFU/rat); 2) 100 mg/kg *S. salivarius* K12 freeze dried powder ($1.67x10^9$ CFU/rat; 3) 5,000 mg/kg *S. salivarius* K12 powder ($8x10^{10}$ CFU/rat); or 4) lyoprotectant control (maltodextrin, trehalose, lactulose). After the 28-day treatment interval all animals were followed for an additional 28 day observation and euthanized on day 56. A fifth group containing 5 males and 5 females was euthanized on day 0 and used for baseline measures. Overall, the S. salivarius K12 treatment was well tolerated by the animals and no clinical evidence of toxicity or pathogenicity were observed. The authors' reported that "*none of the test or control groups of animals demonstrated any detectable health change throughout the 28-day test period*." Gross pathological findings were unremarkable. No statistically significant between group differences in biochemistry or hematology measures were reported at any time-point. However, the Expert Panel noted that AST and ALT values were elevated several-fold above the CCAC[1] historical reference ranges cited in the study in the baseline animals at day 0 and 56. Creatine levels also were elevated at baseline above historical control ranges on these days. Similar elevations outside the reference ranges for AST and ALT were observed in all groups, including control animals, on day 56. These findings are suggestive that animals used in the study may have been in poor health or that validated methods for measurement of clinical chemistry analyses were not employed by the laboratory. On day 28, a blood sample from each animal was plated on blood agar to identify evidence of bacterial translocation. A positive *S. salivarius* K12 culture was reported for 1 high-dose female; however, based on the absence of similar findings in all other treatment animals and the absence of histopathological evidence for vegetative endocarditis in the animal, the authors attributed the findings to potential contamination the blood sample from perforation of the esophagus or respiratory tract during intracardiac bleeding. The Panel considered this conclusion to be reasonable, and further noted that measurements of bacterial translocation in animals administered live microorganisms is a non-validated endpoint, and is of unclear significance given that innocuous translocation of indigenous microflora from the gut to extra-intestinal sites is a normal occurrence that has been reported in studies conducted using healthy rodents (Gordon *et al.*, 1955; Hale and Hill, 1973; Berg, 1983; Steffen and Berg, 1983; Ma *et al.*, 1990; Swank and Deitch, 1996); furthermore, translocation of "probiotic" *Lactobacillus* strains, a genus widely regarded as non-pathogenic also has been reported (Perdigon *et al*., 1997; Rodriguez *et al.*, 2001). The study investigators concluded that "*No abnormal finding occurred for any of the animals on test. Of the parameters measured, which included haematology, biochemistry, urinalysis, organ weights, body weights, clinical observations, none of these provided any evidence of an adverse effect as a result of being treated by repeated dosing with the test substance at three dosages as a sub-acute*

[1] Canadian Council on Animal Care

administration in feed." The Expert Panel identified a number of deviations from GLP and OECD compliance[2] and noted that "*it is generally not possible to identify a concentration of an introduced organism with a level of effect*..." (Suter, 1985); therefore derivation of a no-observed-adverse-effect level (NOAEL) for use in the risk assessment was considered inappropriate. The Panel also noted that *S. salivarius* is a species unique to humans and concluded that information derived from animal toxicity studies conducted using *S. salivarius* K12 are of limited usefulness to the safety assessment. The Panel unanimously concluded that the safety assessment should place an emphasis on well designed human safety studies.

Genotoxicity and Mutagenicity

Genotoxicity testing of *S. salivarius* K12 freeze-dried powder was conducted using the Ames test at concentrations of up to 5 mg/plate (Burton *et al.*, 2010); no evidence of mutagenicity was reported. The Panel noted that additional genotoxicity testing was not necessary to conclude that *S. salivarius* K12 is non-mutagenic and non-genotoxic given that *S. salivarius* K12 is a human isolate, and that *S. salivarius* sp., are ubiquitous and numerically dominant human commensals, which reside within the oral cavity throughout the lifetime of all individuals.

Human Studies

The safety of *S. salivarius* K12 consumption by humans was evaluated in a randomized placebo controlled parallel group study by Burton *et al*. (2011). This study was specifically designed to assess safety and tolerance of *S. salivarius* K12 and included a total 56 healthy male and female adults (aged 20 to 60 years). Participants were randomly allocated to 1 of 2 groups administered $1.1x10^{10}$ CFU/day of *S. salivarius* K12 freeze dried powder or placebo for a period of 28 days. Clinical chemistry, hematology and urinalysis measures were obtained at baseline and on day 28. Gastrointestinal tolerance was monitored using a visual analog scale, and saliva samples were obtained for quantitative PCR detection of *S. salivarius* K12. Consumption of *S. salivarius* K12 was reported to be well-tolerated, and no significant differences in adverse events, physical examination outcomes, or safety parameters (clinical chemistry and hematology) were reported between the placebo and treatment groups. The Expert Panel noted that the study was conducted in accordance with ICH GCP guidelines, and evaluated recognized toxicity safety parameters as primary endpoints. The study was adequately powered and incorporated a double-blinded randomized placebo controlled study design. *S. salivarius* K12 freeze dried powder administered during the study was representative of the commercial product and quantities administered during the trial were an order of magnitude above those occurring from consumption of a single serving of food products to which *S. salivarius* K12 may be added. A NOAEL of $1.1x10^{10}$ CFU/day, the highest dose administered, could be determined from the study. Although the duration of the study at

[2] *E.g*., use of mature (>500 g) rats, pooling of urinalysis samples, pooling of male and female data; analyses of primary toxicity endpoints following a 28-day recovery period]

28 days would not be considered long-term, the Expert Panel recognized that the species is a natural and dominant organism of the oral cavity of all humans and therefore the safety of chronic exposure to the species is well established. The safety of exposure to *S. salivarius* K12 from intended food uses are largely tolerance related and the study design by Burton *et al*. (2012) was considered of sufficient quality to assess this concern.

The administration of *S. salivarius* K12 freeze dried powder to a group of healthy children with and without recent diagnosis of recurrent oral *Streptococcal* pharyngitis/tonsillitis was evaluated by Di Pierro *et al*. (2012) in 82 children 3 to 12 years of age. Sixty-five (65) children with recurrent pharyngitis/tonsillitis were administered single slow-release tablets (n=45) providing $5x10^9$ CFU of *S. salivarius* K12 per child prior to bed once daily. Control subjects (n=20) received no treatment, but were similarly followed throughout the 90 day treatment period. Seventeen (17) children without a history of recurrent pharyngitis/tonsillitis were selected as an additional non-treatment control group. Sixteen (16) subjects from the recurrent-treated group and 14 subjects from the recurrent non-treated group were enrolled for follow-up lasting an additional 6 months during which time the product was not administered. Safety information was obtained as secondary endpoints and included monitoring of tolerability and side-effects during the 90 days of treatment. The authors reported that the *S. salivarius* K12 tablets were well-tolerated and "devoid of side-effects." The Expert Panel noted that the study did not incorporate a randomized placebo controlled study design, was conducted in an unhealthy subject population (*i.e*., recurrent oral pathologies), and therefore was limited as corroborative evidence in the safety assessment.

The consumption of slow release tablets containing *S. salivarius* K12 freeze-dried powder has been evaluated in adults and children with oral *Streptococcal* disorders by Di Pierro and colleagues (Di Pierro *et al*., 2013, 2014). The first study was conducted in a group of 40 adults with recurrent oral *Streptococcal* pharyngitis (Di Pierro *et al*., 2013). Subjects between the ages of 18 and 65 years with total absence of symptoms of infective disease at the time of enrolment were randomized to a treatment or a non-treatment group. Subjects within the treatment group (n=20) were administered a single slow release tablet containing $5x10^9$ CFU *S. salivarius* K12 once daily before bedtime for a period of 90 days. At the end of the 90-day treatment period, 16 subjects from the treatment group and 17 of the non-treated subjects were followed for an additional 6 months during which time the product was not administered. Tolerability, compliance, and side-effects were evaluated as secondary measures during the 90-day treatment period. *S. salivarius* K12 treatment was well tolerated with 19 subjects reporting tolerance as "very good" and one subject reporting tolerance as "good". Side-effects were reported as "none" for these individuals. The second was a multi-center non-randomized controlled study conducted in 61 children 3 to 13 years of age with recurrent oral streptococcal pharyngotonsillitis trial. Thirty-one (31) children received *S. salivarius* K12 slow-dissolve tablets once daily before bed providing at least $1x10^9$ CFU/day for a period of 90-days. Control children were followed up in a similar fashion to treatment subjects but were not provided any treatment.

Thirty (30) of the 31 children in the treatment group reported tolerance as “excellent” and one child reported tolerance as unacceptable due to the taste. None of the children reported side-effects. The Expert Panel considered the studies by Di Pierro *et al*. (2013, 2014) to be corroborative of safety, but limitations in the study design and use of an unhealthy study population limit the value of these studies to the safety assessment.

In a multi-center randomized placebo controlled parallel arm study, Cohen *et al*. (2013) evaluated the administration of a probiotic/prebiotic infant formula preparation containing *S. salivarius* K12 in young infants at increased risk of acute otitis media. Two-hundred and twenty four (224) healthy term infants between the ages of 7 to 13 months participated in the study. Participants were randomized to 1 of 2 treatment groups administered an infant formula containing *S. salivarius* K12 ($5x10^7$ CFU/g per 100 g formula), $2x10^7$ CFU/g, *S. thermophilus*, *Lactobacillus rhamnosus* LPR CGMCC 1.3724 ($2x10^7$ CFU/g per 100 g formula), and fructooligosaccharides (1.9 g per 100 g formula) or placebo formula. Parents of infants were instructed to administer a minimum of 300 mL and not more than 600 mL of the test infant formulas to their respective children on a daily basis for a period of 12 months. Children were estimated to consume between $1x10^9$ and $2x10^9$ CFU *S. salivarius* K12 per day. Anthropometric measurements, general physical examination and otoscopy were obtained at baseline and every 2 months until completion of the trial. The authors reported that the treatment formulas were well-tolerated by the infants and no adverse effects on infant growth were reported. A total of 876 adverse events were reported and 93.1% were determined to be not related to treatment. Only 1 adverse event (constipation) was considered related to treatment. Long-term administration of the test formula also did not result in statistically significant differences in nasopharyngeal composition. The study was adequately powered to measure clinically relevant changes in anthropometric growth indices, a sensitive measure of infant health, and adverse event monitoring demonstrated that the ingredient was well-tolerated. Therefore a NOAEL of $1x10^9$ CFU/day, the lower range of the formula volume consumed per day, could be derived for the study. This intake is comparable to that expected from consumption of multiple servings of *S. salivarius* K12 containing foods, which will contain up to $1.0x10^9$ CFU/serving, and on a per kg basis would greatly exceed intakes occurring from food uses.

The Panel also reviewed published findings from several additional controlled and uncontrolled studies have been conducted with *S. salivarius* K12 in healthy and unhealthy adults and children. No adverse events were reported in children requiring ventilation tube placement surgery who were fed a powdered formulation providing up to $3.4x10^{10}$ CFU/day of *S. salivarius* K12 for a period of 10 days (Power *et al.*, 2008). *S. salivarius* K12 also was reported to be well-tolerated in clinical studies wherein healthy adults ingested up to $4x10^{10}$ CFU of *S. salivarius* K12/day for periods ranging from 3 to 14 days (Burton *et al.*, 2006a,b; Horz *et al.*, 2007; Burton *et al*., 2010; Lee *et al.*, 2010). Findings from these studies were corroborative of safety;

however, limitations in the study designs and study populations limit their use in safety assessment.

Assessment of Pathogenic and Toxigenic Potential

A comprehensive search of the literature was conducted by BLIS using the search terms *Streptococcus* AND salivarius AND: disease, infection, pathogenicity, toxicity, infectivity, illness, cancer, meningitis, endocarditis, respiratory, bacteremia, and impetigo. All relevant articles were reviewed and summarized. Several case-reports of opportunistic infections in association with surgical procedures, tissue trauma, or predisposing medical conditions (*e.g*., cancer, organ transplant, tonsillitis) were identified. With the exception of cases of iatrogenic meningitis for which *S. salivarius* strains appear to be the most common cause, reports of pathogenic infections attributed to *S. salivarius* were identified as opportunistic in nature, and were comparable to those reported for members of the *Lactobacillus* genus, which have long-histories of safe use in food (Borriello *et al*., 2003). To date, genotypic and bioinformatic analyses of *S. salivarius* K12, and other *S. salivarius* isolates, including opportunistic isolates, have not identified pathogenicity determinants of viridians *Streptococci* (Burton *et al*., 2006a; Delorme *et al*., 2007, 2011; Barretto *et al*., 2012). Cases of iatrogenic meningitis associated with *S. salivarius* were not considered relevant to oral exposures occurring from use in food.

An independent review of the use of *S. salivarius* K12 in food within the context of the available data and information relevant to reports of human infections associated with *S. salivarius* was conducted by Dr. Jonathan R Carapetis (Ph.D., Director, Menzies School of Health Research, University of Melbourne). Dr. Carapetis is a medical practitioner with qualifications in pediatric infectious diseases and public health, and is a qualified Expert in *Streptococcal* disease (Carapetis *et al*., 2005). Following a review of the aforementioned information, 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 that other commonly used probiotic organisms*."

The 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 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 (Kort *et al*., 2014). Available evidence also indicates that *S. salivarius* K12 is not able to colonize the gut mucosa. Based on these conclusions and the aforementioned data and information, the Panel similarly concluded that the species *S. salivarius* is non-pathogenic, and that the risk of opportunistic infection from the proposed use of *S. salivarius* K12 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., marketplace.

Antibiotic Resistance

The antibiotic resistance profile of a microorganism is a strain specific phenotype, and *in vitro* antibiotic resistance testing has been conducted for *S. salivarius* K12 against a broad range of clinically important antibiotics. Based on observations from a number of *in vitro* experiments, *S. salivarius* K12 has been shown to be sensitive to antibiotics routinely used for the control of upper respiratory tract infections. *In vitro* antibiotic resistance testing of successive generations indicated that the antibiotic resistance profile of the strain was stable. Using microdilution and disk diffusion methods, antibiotic resistance was only observed against compounds within the aminoglycoside family of antibiotics, and notable findings were possible evidence for resistance to kanamycin.

Bioinformatic Assessment

Finally, a bioinformatic search of the annotated gene sequence was conducted to identify any potentially undesirable phenotypic properties of the organism. No genes involved in pathogen adhesion, invasion, toxin or superantigen production, or regulation of virulence were identified in the analysis. In addition, no plasmid-borne antibiotic resistance genes, and no point mutations in genes associated with antibiotic resistance were identified. Several putative antibiotic resistance genes were identified; however, in conjunction with the existing *in vitro* evidence, confirmation of antibiotic resistance genes could only be supported for the aminoglycosides (*i.e.*, kanamycin). Although *S. salivarius* K12 is strongly suspected to harbor aminoglycoside antibiotic resistance determinants, due to the chromosomal location of the gene, and the fact that no gene sequences matching known transposable elements were identified in close proximity to any of the annotated genes, the Panel noted that the likelihood of this antibiotic resistance being transferred to other Streptococcal or closely-related species is low.

CONCLUSION

We, the members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to safety of the intended uses of *Streptococcus salivarius* K12. We unanimously conclude that the intended uses of *Streptococcus salivarius* K12, meeting appropriate food-grade specifications presented in the supporting dossier and manufactured consistent with current Good Manufacturing Practice (cGMP), are safe and suitable.

We further unanimously conclude that the intended uses of *Streptococcus salivarius* K12, meeting appropriate food-grade specifications presented in the supporting dossier and manufactured consistent with current Good Manufacturing Practice (cGMP), are Generally Recognized as Safe (GRAS) based on scientific procedures.

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

(b) (6)

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APPENDIX A

Individual Proposed Food-Uses and Use-Levels for Freeze-Dried *Streptococcus* K12 Powder in the U.S.

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Streptococcus salivarius* K12 in the United States (NHANES 2003-2006)

Food Category	Proposed Food-Uses	*S. salivarius* K12 Use-Level (mg/ serving)	Serving Size (g or mL)*	Use-Level (%)
Baby and Toddler Foods	Cereals, Baby Food	20	15 (dry, instant)[a] 110 (RTS)[a]	0.13 (dry, instant) 0.018 (RTS)
	Cookies, Crackers, and Puffs, Baby/Toddler Food	20	7[a]	0.10
	RTS Fruit-Based Baby/Toddler Food	20	60 (strained)[a] 110 (junior)[a] 125 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.016 (toddler)
	Fruit Juices, Baby Food	20	125[a]	0.016
	RTS Dinners, Baby/Toddler Food	20	60 (strained)[a] 110 (junior)[a] 170 (toddler)[a]	0.03 (strained) 0.018 (junior) 0.012 (toddler)
	RTS Desserts, Baby Food	20	60 (strained) 110 (junior)	0.03 (strained) 0.018 (junior)
	RTF Vegetable-Based Baby/Toddler Food	20	60 (strained) 110 (junior) 70 (toddler)	0.03 (strained) 0.018 (junior) 0.029 (toddler)
Baked Goods and Baking Mixes	Cookies (chocolate coating)	20	20	0.10
Beverages and Beverage Bases	Meal Replacement powders (fortified, protein, and mineral replenish)	20	16 to 40	0.05 to 0.13
	Sports and Energy Drinks	20	250	0.01
	Water (Still or Mineral)	20	237	0.01
Breakfast Cereals	Breakfast Cereals	20	29	0.07
	Muesli and Dry Blended Cereals	20	85	0.02
Cheeses	Natural Cheeses	20	20 to 30	0.07 to 0.10
Chewing Gum	Chewing Gum	20	3	0.67
Dairy Product Analogs	Milk Substitutes	20	244	0.01
Frozen Dairy Desserts and Mixes	Frozen Yogurt	20	174	0.02
	Ice Cream	20	66	0.03
Gelatins, Puddings, and Fillings	Custards (pourable)[b]	20	113	0.02
	Dessert Mixes (powder)	20	25	0.08
Grain Products and Pastas	Granola and Breakfast Bars	20	28	0.07
	Protein Bars	20	68	0.03
Hard Candy	Mint Candies	20	25	0.08
Milk, Whole and Skim	Milk (flavored, pasteurized)	20	244	0.01
	Milk (fresh)	20	244	0.01
	Milk Powder (skim or whole)	20	23 to 32	0.06 to 0.09

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Streptococcus salivarius* K12 in the United States (NHANES 2003-2006)

Food Category	Proposed Food-Uses	*S. salivarius* K12 Use-Level (mg/ serving)	Serving Size (g or mL)*	Use-Level (%)
Milk Products	Cream (pasteurized)	20	244	0.01
	Cultured Milk Products	20	180	0.01
	Dairy Desserts	20	100 to 180	0.01 to 0.02
	Milkshake Mixes (powder)	20	21	0.10
	Yogurt	20	227	0.01
	Yogurt Drinks	20	244	0.01
Nuts and Nut Products	Peanut Butter	20	32	0.06
Processed Fruits and Fruit Juices	Fruit-Flavored Beverages (powder)	20	18	0.11
	Fruit Juices	20	263	0.01
	Fruit Juice Drinks	20	209	0.01
Soft Candy	Chewable Lozenges[c]	20	3	0.67
	Soft Gel and Rapid Melt Technologies[d]	20	2	1
Sweet Sauces, Toppings, and Syrups				
	Sugar and Sweetener Sprinkle	10	4[a]	0.25

RTD = ready to drink; RTE = ready to eat; 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 U.S. CFR (21 CFR §101.12) (U.S. FDA, 2012).
[b] No food codes for custard (pourable) is available in NHANES 2003-2006; however, surrogate codes (custard-filled products) that have a similar composition and pattern of use will be used to represent this category.
[c] No food codes for chewable lozenges is available in NHANES 2003-2006; however, the exposure to chewable lozenges is expected to be similar to the exposure from mint candies, which is already included as one of the proposed food-uses.
[d] No food codes for soft gel and rapid melt technologies were identified in NHANES 2003-2006.

SUBMISSION END