Dose Biosystems Inc.
661 University Ave, Suite 1300
Toronto, ON M5G 0B7 CANADA

Submission Date: June 7, 2021

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

Attn: Susan J. Carlson, Ph.D., Director, Division of Food Ingredients

Dear Dr. Carlson,

Pursuant to 21 CFR Part 170, Subpart E, Dose Biosystems Inc. hereby submits a Generally Recognized as Safe (GRAS) notice for *Streptococcus salivarius* DB-B5. Dose Biosystems has concluded that *S. salivarius* DB-B5 is GRAS under its intended conditions of use as an ingredient in conventional foods in the United States (U.S.), based on scientific procedures.

As a species, *S. salivarius* and the closely related *S. thermophilus* (previously *S. salivarius* subsp. *thermophilus*) have a history of safe use as starter cultures in fermented foods. The U.S. Food and Drug Administration (FDA) has also issued "no questions" responses regarding the conclusions that *S. salivarius* K12 (GRN No. 591) and *S. salivarius* M18 (GRN No. 807) are Generally Recognized as Safe (GRAS) for their intended uses in foods.

Please do not hesitate to contact me should you require any clarifications regarding this GRAS notice. We look forward to hearing from you.

Sincerely,

Mizue Naito, Ph.D.
Director, Probiotics & Microbiome R&D
Dose Biosystems Inc.
mizue@dosebiosystems.com
(+1) 416-986-3750

Generally Recognized as Safe (GRAS) Notice for *Streptococcus salivarius* DB-B5

Dose Biosystems Inc.
MaRS Discovery District
661 University Ave, Suite 1300
Toronto, ON
M5G 0B7, Canada

07 June 2021

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1. Signed Statements and Certifications (21 CFR §170.225)

1.1 Regulatory Citation

Dose Biosystems Inc. ("Dose Biosystems") submits this Generally Recognized as Safe (GRAS) notice to the United States (U.S.) Food and Drug Administration (FDA), in accordance with 21 CFR Part 170, Subpart E.

1.2 Name and Address

Dose Biosystems Inc.
MaRS Discovery District
661 University Ave, Suite 1300
Toronto, ON
M5G 0B7, Canada

1.3 Name of Notified Substance

Streptococcus salivarius DB-B5

1.4 Intended Conditions of Use

Dose Biosystems intends to use *S. salivarius* DB-B5 as a general ingredient in conventional foods at target levels providing a minimum of $1x10^9$ CFU/serving. *S. salivarius* DB-B5 is not intended for addition to infant formula, or to meat and poultry products that are subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA).

1.5 Statutory Basis for GRAS

The conclusion of GRAS status for the intended uses of *S. salivarius* DB-B5 is made through scientific procedures, in accordance with 21 CFR §170.30 (a) and (b).

1.6 Exemption from Premarket Approval

Dose Biosystems has concluded their *S. salivarius* DB-B5 strain is GRAS under its intended conditions of use, and as such, it is not subject to the premarket approval requirements in the Federal Food, Drug, and Cosmetic Act.

1.7 Availability of Data and Information

Dose Biosystems agrees to make the data and information that serve as the basis for the GRAS conclusion of *S. salivarius* DB-B5 available to the FDA upon request. Dose Biosystems will allow the FDA to review and copy the data and information during customary business hours at the address indicated

in Section 1.2 above. Alternatively, Dose Biosystems will provide the FDA with a complete copy of the data and information either in an electronic format that is accessible for the FDA's evaluation, or on paper.

1.8 FOIA STATEMENT

The data and information presented in Parts 2 through 7 of this notice do not contain any trade secret, commercial, or financial information that are privileged or confidential. Therefore, none of the data and information presented herein are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. Section 552.

1.9 FSIS STATEMENT

Not applicable. The intended conditions of use for *S. salivarius* DB-B5 does not include uses in product or products that are subject to regulation by the FSIS.

1.10 CERTIFICATION AND SIGNATURE

To the best of Dose Biosystems' knowledge, this GRAS notice is a complete, representative, and balanced compilation that includes all relevant information, both favorable and unfavorable, that are pertinent to the evaluation of the safety and GRAS status of *S. salivarius* DB-B5 under its intended conditions of use.

Signature of Notifier:

[redacted]

June 7, 2021

Mizue Naito
Director, Probiotics & Microbiome R&D
Dose Biosystems Inc.

Date

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECTS (21 CFR §170.230)

2.1 IDENTITY

Common name: *Streptococcus salivarius* DB-B5

Taxonomical Lineage:

Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Streptococcaceae
Genus:	*Streptococcus*
Species:	*salivarius*
Strain:	DB-B5

2.1.1 Source of *S. salivarius* DB-B5

The oral cavity houses one of the most diverse microbiota in the human body. There are nearly 800 unique oral bacterial species identified in the Human Oral Microbiome Database (Chen *et al.*, 2010), with more species expected to be added with further sampling and identification. *S. salivarius* is a pioneer species that colonizes the human oral cavity from birth, and it remains a predominant member of the commensal oral microbiota throughout life (Wescombe *et al.*, 2012). *S. salivarius* DB-B5 was isolated from the supragingival plaque of a healthy female adult donor (Fields *et al.*, 2020), and it has been deposited at the International Depository Authority of Canada. The strain is not genetically engineered.

2.1.2 Genotypic Identification

2.1.2.1 Genetic Similarities between S. salivarius and S. thermophilus

S. salivarius is placed under the "Salivarius group" of viridans Streptococci, which also includes *S. thermophilus* and *S. vestibularis* (Burton *et al.*, 2017). *S. vestibularis* is a human commensal like *S. salivarius*, and *S. thermophilus* is a species widely used as starter cultures for fermented foods such as yogurts (Burton *et al.*, 2017). There is a high degree of genetic similarity between *S. salivarius* and *S. thermophilus* (*i.e.*, 99% at the 16S rRNA gene level) (Burton *et al.*, 2017). An in-depth discussion of the genetic relatedness between *S. salivarius* and *S. thermophilus* has been previously presented in the GRAS notices for *S. salivarius* K12 (GRN No. 591) and *S. salivarius* M18 (GRN No. 807).

In brief, the high degree of genetic similarity has led to the contention of whether *S. thermophilus* is a distinct species from *S. salivarius*, or if it should be considered a subspecies of *S. salivarius*. Originally, *S.*

thermophilus was recognized as a species on its own right by Orla-Jensen in 1919 (ITIS, 2012). However, in 1984, Farrow and Collins demonstrated that *S. thermophilus* and *S. salivarius* exhibited a similar GC content (37 to 41%), had a comparable long-chain fatty acid profiles, and belonged to a single DNA homology group based on DNA-DNA hybridization experiments (Farrow & Collins, 1984). Thus, it was proposed that *S. thermophilus* should be more appropriately classified as *S. salivarius* subsp. *thermophilus* (Farrow & Collins, 1984). Subsequently, Shleifer and colleagues conducted further DNA-DNA hybridization experiments and concluded that *S. thermophilus* deserved separate full species status, and that its name should be reverted to its former one (Schleifer *et al.*, 1991). More recent phylogenetic analyses also suggest that *S. thermophilus* and *S. vestibularis* descended from a common ancestor subsequent to the early divergence of *S. salivarius*, further supporting that the 3 are taxonomically distinct but closely related species (Delorme *et al.*, 2015; Martinović *et al.*, 2020; Pombert *et al.*, 2009). However, the nomenclature has not been fully ratified by taxonomic committees, and the species is still widely reported as *S. salivarius* subsp. *thermophilus* in the literature (Burton *et al.*, 2017). As stated in GRN No. 807, *"the close genetic relationship between S. salivarius and S. thermophilus, and the long-history of safe use of S. thermophilus in yogurt starters strongly supports the contention that the evolution of pathogenic traits has not occurred in this lineage."*

2.1.2.2 Phylogenetic Reconstruction of S. salivarius DB-B5

The taxonomic placement of *S. salivarius* DB-B5 strain has been definitively confirmed using both 16S rRNA and multi-gene phylogenetic reconstruction (Li *et al.*, 2021). The Integrated Microbial Genomes and Microbiomes database (IMG; https://img.jgi.doe.gov/) were used to obtain the non-*S. salivarius* DB-B5 sequences. The multi-gene phylogenetic tree was constructed with the following genes, based on the Genomic Encyclopedia of Bacteria and Archaea project (Wu *et al.*, 2009): *dnaG, frr, infC, nusA, pgk, rplA, rpoB, rpsC, smpB, tsf*. Both Bayesian inference and Maximum Likelihood methods confirmed the placement of *S. salivarius* DB-B5 as a member of the *S. salivarius* species. The 16S rRNA phylogenetic tree and multi-gene phylogenetic tree are presented in Figure 2.1.2.2-1 and Figure 2.1.2.2-2, respectively.

Figure 2.1.2.2-1 **Phylogenetic Reconstruction of *S. salivarius* DB-B5 Using 16S rRNA**

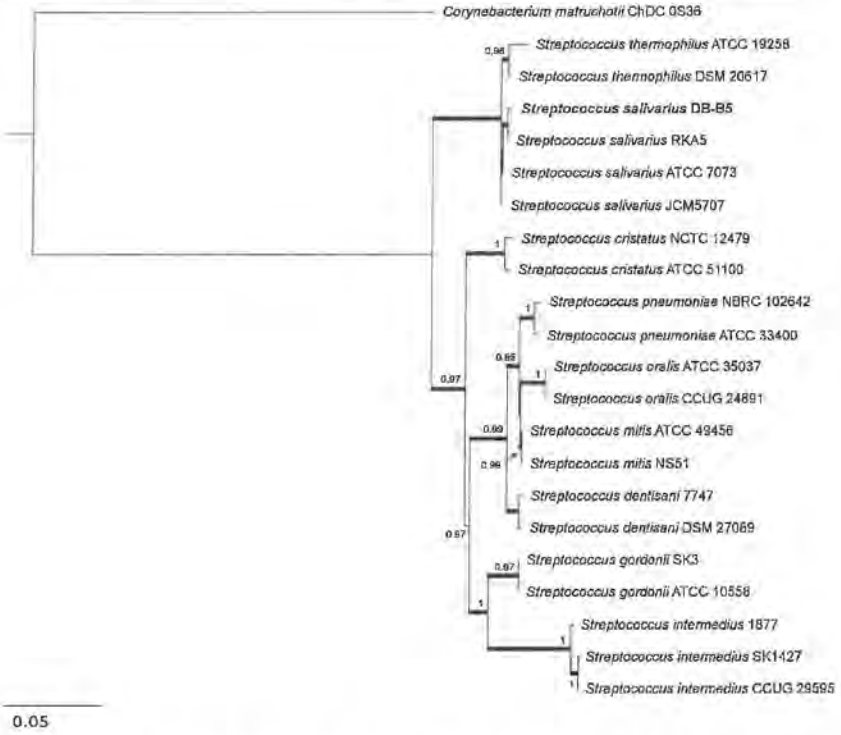
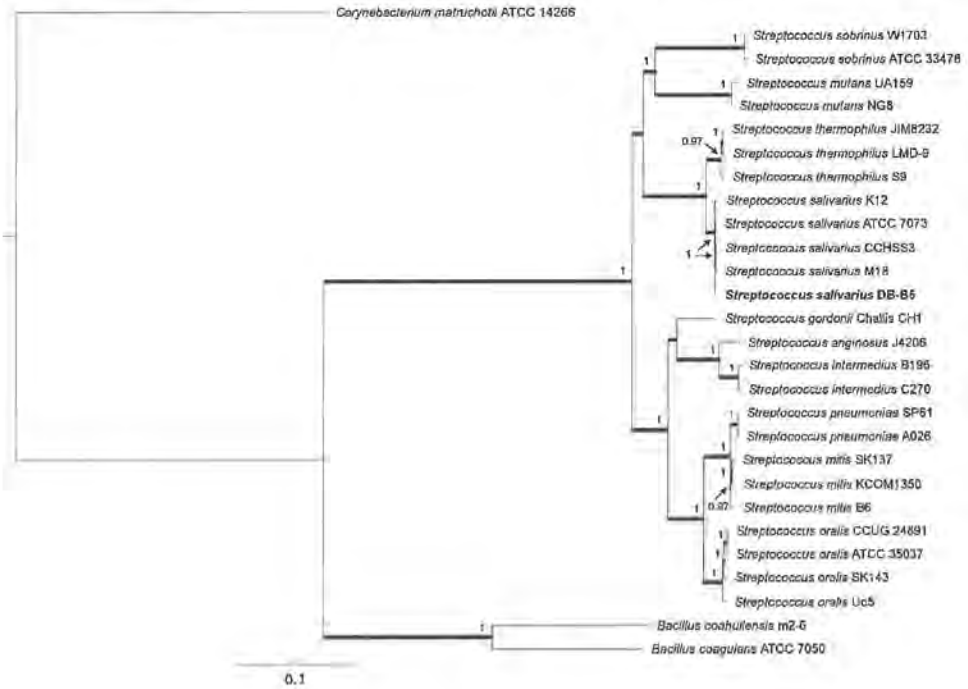


Figure 2.1.2.2-2 **Phylogenetic Reconstruction of *S. salivarius* DB-B5 Using Multi-Gene Analysis**



2.1.2.3 Whole Genome Sequencing

The genome of *S. salivarius* DB-B5 has been fully sequenced, assembled, and annotated. Details of the whole genome sequencing methodology have been published (Fields *et al.*, 2020). In brief, genomic DNA was extracted and sequencing was performed using a hybrid assembly approach by combining Illumina MiSeq short reads and PacBio long reads.

The complete genome consists of one circular chromosome (2,143,863 bp) with a GC content of 40.2%, one megaplasmid named pIKMIN-B501 (138,497 bp) with a GC content of 35.6%, one small plasmid named pIKMIN-B503 (3,225 bp) with a GC content of 39.6%, and one linear phage-like element named pIKMIN-B502 (57,714 bp) with a GC content of 39.1%. This is consistent with other *S. salivarius* genomes, which are approximately 2.1 to 2.3 Mb with a GC content of approximately 39 to 40%[1]. The genome of *S. salivarius* DB-B5 was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v4.11. The genome contains a total of 2,041 protein coding genes, 18 complete rRNA genes, 4 non-coding RNA genes (ncRNA), and 68 tRNA genes. The complete genome sequences of *S. salivarius* DB-B5 have been deposited in GenBank® under the accession numbers CP054153 (chromosome), CP054154 (pIKMIN-B501), CP054155 (pIKMIN-B502), and CP054156 (pIKMIN-B503).

2.1.3 Phenotypic Characterization

2.1.3.1 Morphology

Streptococcus salivarius cells are spherical to ovoid in shape, ranging 0.8 to 1.0 µm in diameter, and they typically form chains of varying lengths (Whiley & Hardie, 2015). Morphologically, *S. salivarius* DB-B5 appears similar to *S. salivarius* K12, with single-cell, diplococcic, and longer-chain aggregates observed under microscopy.

2.1.3.2 Carbohydrate Fermentation Profile

The carbohydrate fermentation profile of *S. salivarius* DB-B5 has been determined using the API 50CH test strips from bioMérieux Inc., according to the instructions provided. The API strip containing *S. salivarius* DB-B5 was incubated aerobically at 37°C, and the fermentation profile was assessed at 24 and 48 hours (Li *et al.*, 2021). *S. salivarius* DB-B5 was able to ferment 17 of the 49 carbohydrates tested, and the fermentation profile is comparable to those observed for other commercialized *S. salivarius* strains (see Table 2.1.3.2-1). No unusual metabolic capabilities were observed for *S. salivarius* DB-B5. Dose Biosystems has also verified that the fermentation profile of *S. salivarius* DB-B5 is stable under numerous lab propagations, as well as fermentation and freeze-drying processes (Li *et al.*, 2021).

Table 2.1.3.2-1 Carbohydrate Fermentation Profile of *S. salivarius* DB-B5

Substrate	*S. salivarius* DB-B5[a]	*S. salivarius* K12[a] (GRN No. 591)	*S. salivarius* M18[a] (GRN No. 807)
glycerol	-	-	-
erythritol	-	-	-
D-arabinose	-	-	-

[1] Taken from IMG (https://img.jgi.doe.gov/).

Substrate	*S. salivarius* DB-B5[a]	*S. salivarius* K12[a] (GRN No. 591)	*S. salivarius* M18[a] (GRN No. 807)
L-arabinose	-	-	+ (anaerobic only)
D-ribose	-	-	-
D-xylose	-	-	-
L-xylose	-	-	-
D-adonitol	-	-	-
methyl-β-D-xylopyranoside	-	-	-
D-galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	-	-	-
L-rhamnose	-	-	-
dulcitol	-	-	-
inositol	-	-	-
D-mannitol	-	-	-
D-sorbitol	-	-	-
methyl-α-D-mannopyranoside	-	-	-
methyl-α-D-glucopyranoside	-	-	-
N-acetylglucosamine	+	+	+
amygdalin	+	-	+
arbutin	+	+	+
esculin	+	+	+
salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose	-	+	+
D-melibiose	+	-	+ (aerobic only)
D-saccharose (sucrose)	+	+	+
D-trehalose	+	+	+
inulin	+	+	+
D-melezitose	-	-	-
D-raffinose	+	+	+
amidon (starch)	+/-	-	-
glycogen	-	-	+ (anaerobic only)
xylitol	-	-	-
gentiobiose	+	-	+
D-turanose	-	-	-
D-lyxose	-	-	-
D-tagatose	-	+	+ (anaerobic only)
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
gluconate	-	-	-
2-ketogluconate	-	-	-
5-ketogluconate	-	-	-

[a] Measured with API 50CH test strips. "+" indicates the ability to ferment the carbohydrate.

2.1.3.3 Enzyme Activities

The API 20 Strep test kit from bioMérieux Inc. was used to evaluate the enzyme activity profile of *S. salivarius* DB-B5. The test kit consists of 20 wells containing dehydrated substates, which allows for the determination of specific enzymatic activities, as well as the capacity to ferment certain sugars. The reactions were evaluated following incubation with *S. salivarius* DB-B5 under anaerobic conditions at 37°C for 4 hours for the determination of enzymatic activities, and for 24 hours for the determination of the carbohydrate fermentation capacities (Li *et al.*, 2021). As summarized in Table 2.1.3.3-1, *S. salivarius* DB-B5 exhibits a similar enzymatic activity profile as the commercially available *S. salivarius* K12 and M18 strains.

Table 2.1.3.3-1 Enzyme Activity Profile of *S. salivarius* DB-B5

Substrate	*S. salivarius* DB-B5[a]	*S. salivarius* K12[a] (GRN No. 591)	*S. salivarius* M18[a] (GRN No. 807)
Acetoin production	+	+	+
Hippuric acid hydrolysis	-	-	-
β-Glucosidase	+	+	+
Pyrrolindonyl 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	-	-	-

[a] Assessed using the API 20 Strep test strips. "+" indicates the presence of the enzyme activity listed, and the ability to ferment the carbohydrate tested.

2.1.3.4 Hemolytic Activity

Historically, one of the earliest methods used to differentiate species within the *Streptococcus* genus was through the observation of their hemolysis patterns (Facklam, 2002; Sherman, 1937). The ability of bacteria to lyse red blood cells can be phenotypically evaluated by streaking them on blood agar plates and observing the level of blood lysis surrounding the cells. Beta hemolysis is defined as the complete lysis of the red blood cells, and a clear zone approaching the color and transparency of the base medium is observed on the blood agar plates where the bacteria were spotted (Buxton, 2016). Alpha hemolysis represents partial or incomplete lysis whereby the red blood cell membranes remain intact, but the hemoglobin is oxidized to methemoglobin, resulting in a greenish hue on the plates (Buxton, 2016;

Pradhan *et al.*, 2020). Gamma hemolysis means no lysis of the red blood cells are observed (Buxton, 2016).

Major human streptococcal pathogens belong to the "pyogenic" division of streptococci, which consists largely of species that are beta-hemolytic (de la Maza, L. M. *et al.*, 2020; Lancefield, 1933; Sitkiewicz & Hryniewicz, 2010; Whiley & Hardie, 2015). Examples include *Streptococcus pyogenes* (Group A Streptococcus) and *Streptococcus agalactiae* (Group B Streptococcus) (Abranches *et al.*, 2018; Facklam, 2002; Whiley & Hardie, 2015). The "viridans" division of streptococci on the other hand has historically included the large group of commensal streptococcal Gram-positive bacteria in the oral cavity, including *S. salivarius* (Abranches *et al.*, 2018; Facklam, 2002). The greenish hue on the blood agar plates that results from alpha hemolysis forms the original basis of their name, as "viridans" is derived from the Latin word "vĭrĭdis" meaning green (Abranches *et al.*, 2018; Parks *et al.*, 2015).

The hemolytic activity of *S. salivarius* DB-B5 was assessed using Brucella blood agar with hemin and vitamin K, which contains 5% sheep blood (Li *et al.*, 2021). The plates were incubated overnight at 37°C under 5% CO_2. Beta hemolysis was not observed for *S. salivarius* DB-B5, consistent with the lack of beta hemolytic activity reported for *S. salivarius* K12 and M18 in GRN No. 591 and GRN No. 807. Instead, the area surrounding *S. salivarius* DB-B5 strains on the blood agar plate was found to be a dark green/brown colour, indicative of alpha or partial hemolysis (Figure 2.1.3.4-1). The same phenotype was also observed with *S. salivarius* K12 and *S. salivarius* M18 when it was tested under the same conditions (Figure 2.1.3.4-1).

The presence of alpha hemolysis for *S. salivarius* K12 and *S. salivarius* M18 is contradictory to the results that have been previously reported. In one experiment where *S. salivarius* K12 was tested in 3 different media (human blood agar with 5% v/v human blood, or sheep blood agar or buffered CNA-P agar with 5% defibrinated sheep blood), the study authors reported that *"no hemolytic activity was detected"* (Burton, Wescombe *et al.*, 2006). The absence of hemolytic activity has also been reported for *S. salivarius* M18 in regulatory submissions (TGA, 2019). The discrepancy between these results could be due to differences in the visual interpretation of alpha hemolysis (partial lysis) *vs.* gamma hemolysis (*i.e.*, no lysis). While complete lysis (beta hemolysis) is obvious to the naked eye, alpha and gamma hemolysis are difficult to differentiate from each other. Furthermore, the composition of the medium, including the type and concentration of blood used, as well as the incubation conditions, can influence the extent of hemolysis that occurs (Doern & Burnham, 2010; Facklam, 2002; Patterson, 1996; Whiley & Hardie, 2015).

The presence of alpha hemolysis is not considered to pose any safety concerns for the intended uses of *S. salivarius* DB-B5 as a food ingredient. Most *S. salivarius* strains characteristically display alpha hemolysis but are generally considered safe members of the commensal oral microbiota (de la Maza, L. M. *et al.*, 2020). Alpha hemolysis was similarly observed for the commercially available *S. salivarius* K12 and M18 strains when it was tested under the same testing conditions as *S. salivarius* DB-B5. Evidence of alpha hemolysis have also been reported from microbials that are food isolates or are used for technological functions in food (*e.g., S. thermophilus*, lactobacilli) (Adimpong *et al.*, 2012; Goldstein *et al.*, 2015; Maragkoudakis *et al.*, 2006; Pradhan *et al.*, 2020; Schleifer *et al.*, 1991; Siegrist, Unknown). No toxigenic effect has been documented as a by-product of alpha hemolysis (Doern & Burnham, 2010), and as described further in Section 6.5.2, bioinformatic analyses have demonstrated the genome of *S. salivarius* DB-B5 does not harbor any potential virulence factors of concern (*e.g.*, hemolysins).

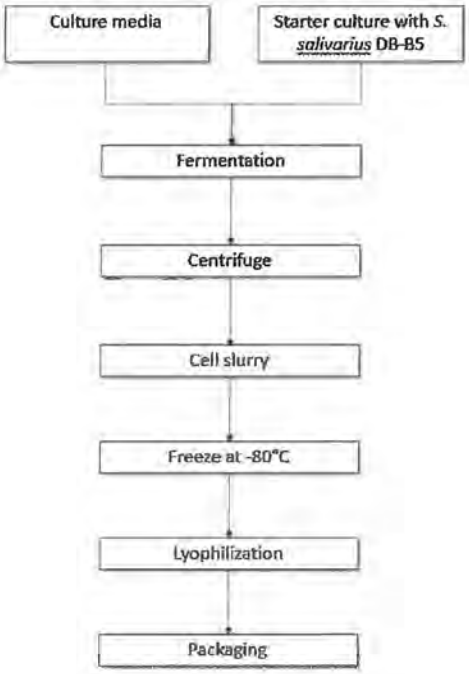
Figure 2.1.3.4-1 **Hemolysis Assay for *S. salivarius* DB-B5 and Other Commercially Available Strains with GRAS Status in the U.S. (*S. salivarius* K12 and M18)**

Note: Image is representative of three biological replicates, performed in triplicates.

2.2 METHOD OF MANUFACTURE

The manufacturing process of *S. salivarius* DB-B5 is conducted in accordance with current Good Manufacturing Practice (GMP), and a Hazard Analysis Critical Control Points (HACCP) system is in place to ensure the production of a high-quality product. A flowchart of the manufacturing process is presented in Figure 2.2-1.

Figure 2.2-1 Schematic Overview of the Manufacturing Process for *S. salivarius* DB-B5



The *S. salivarius* DB-B5 master cell bank (MCB) is maintained in frozen vials stored at -80°C in Dose Biosystem's facilities. The MCB is subjected to quality control testing to confirm the identity of the *S. salivarius* DB-B5 strain and to ensure it is free from microbiological contaminants. The MCB is used to derive the working cell banks which are used to manufacture production lots of *S. salivarius* DB-B5.

The production process of *S. salivarius* DB-B5 begins with its addition into a defined culture medium. The culture medium is composed of growth substrates, namely a mixture of carbohydrates, amino acids, vitamins and minerals, as well as technological aids. Both the culture medium and the cryoprotectants are sterilized prior to use. Fermentation of *S. salivarius* DB-B5 takes place under anaerobic conditions at controlled pH and temperature, within a contained and sterile environment. Once microbiological growth has reached the desired level, the fermentation process is stopped, and the cells are harvested by centrifugation and filtration. The resulting cell slurry is mixed with cryoprotectants and frozen at -80°C, following which it is freeze-dried in a lyophilizer. The lyophilized *S. salivarius* DB-B5 powder is then packaged and sealed for storage.

All of the materials employed in the manufacture of *S. salivarius* DB-B5 (*i.e.*, fermentation medium components, cryoprotectants) are food-grade and suitable for use in the U.S., meeting the specifications set forth in the Food Chemicals Codex, or their equivalent international food or pharmacopeia standards. When applicable, finished food products containing *S. salivarius* DB-B5 will be labeled with appropriate allergen declarations (*e.g.*, soy), as required under the *Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004* amending the *Federal Food, Drug, and Cosmetic Act*.

2.3 PRODUCT SPECIFICATIONS AND BATCH ANALYSES

2.3.1 Specifications

Dose Biosystems has established food-grade specifications for *S. salivarius* DB-B5, which are presented in Table 2.3.1-1 below. In addition to establishing parameters for strain identification and quantification, the specifications set forth acceptable limits for microbiological and heavy metal contaminants, which are measured using recognized and validated methods of analysis.

Table 2.3.1-1 Product Specifications for *S. salivarius* DB-B5

Parameter	Specification	Method of Analysis
Characteristics		
Appearance	White to off-white powder	Visual observation
Identity	Confirmed	16S rRNA
Enumeration (CFU/g)	NLT $1x10^{10}$	Internal method
Microbiological Criteria		
Aerobic plate count (CFU/g)	NMT 50	USP<61>
Yeast and mold count (CFU/g)	NMT 50	USP<61>
Salmonella	Negative	USP<62>
Escherichia coli	Negative	USP<62>
Bile tolerant gram-negative bacteria	Negative	USP<62>
Heavy Metals		
Cadmium (mg/kg)	<0.1	ICP-MS
Lead (mg/kg)	<0.3	ICP-MS
Mercury (mg/kg)	<0.1	ICP-MS
Arsenic (mg/kg)	<0.1	ICP-MS

CFU = colony forming units; NLT = not less than; NMT = not more than.

2.3.2 Batch Analyses

Analytical data from 3 representative non-consecutive manufacturing lots of *S. salivarius* DB-B5 are presented in Table 2.3.2-1. These data provide support that the manufacturing process produces a consistent material that meets the specifications defined above in Section 2.3.1.

Table 2.3.2-1 Analytical Data from 3 Representative Lots of *S. salivarius* DB-B5

Parameter	Specification	Lot Number		
		BR-PD-5	BR-PD-6	BR-PD-7
Characteristics				
Appearance	White to off-white powder	Conforms	Conforms	Conforms
Identity	Confirmed	Confirmed	Confirmed	Confirmed
Enumeration (CFU/g)	NLT $1x10^{10}$	$3.17 x10^{10}$	$1.22 x10^{10}$	$5.40 x10^{10}$
Microbiological Criteria				
Aerobic plate count (CFU/g)	NMT 50	<10	<10	<10
Yeast and mold count (CFU/g)	NMT 50	<10	<10	<10
Salmonella	Negative	Negative	Negative	Negative
Escherichia coli	Negative	Negative	Negative	Negative

Parameter	Specification	Lot Number		
		BR-PD-5	BR-PD-6	BR-PD-7
Bile tolerant gram-negative bacteria	Negative	Negative	Negative	Negative
Heavy Metals				
Cadmium (mg/kg)	<0.1	0.015	0.017	0.026
Lead (mg/kg)	<0.3	0.159	0.074	0.076
Mercury (mg/kg)	<0.1	<0.010	<0.010	<0.010
Arsenic (mg/kg)	<0.1	0.018	0.016	0.019

CFU = colony forming units; NLT = not less than; NMT = not more than.

2.4 STABILITY

Dose Biosystems is currently conducting studies to investigate the stability of *S. salivarius* DB-B5 during bulk storage for up to 24 months. Lyophilized *S. salivarius* DB-B5 is kept in its sealed packaging at refrigerated (5°C ± 3°C) and controlled room temperature (25°C ± 2°C, at 60±5% relative humidity (RH)). Aliquoted samples are taken for measurements at baseline, 1, 2, 3, 6, 9, 12, 18, and 24 months. The data obtained to date demonstrate that *S. salivarius* DB-B5 is stable for at least 12 months when kept in storage at either refrigerated or room temperature, with normal levels of gradual loss in viability (around 1 log over a 12 month period) when using CFU counts. While one data point (6 months) indicated an unusual drop in CFU/g, this data point appears to be an anomaly, as the 9 and 12 month time points were above 10^{10} CFU/g. Stability using fluorescence microscopy (TCC/g + viability %) indicates that *S. salivarius* DB-B5 is stable for 12 months, without the viability loss seen using CFU counts.

Table 2.4-1 Stability Data for *S. salivarius* DB-B5 Stored at 5°C and 25°C

Parameter	Time in Storage (months)						
	0	1	2	3	6	9	12
Storage at 5°C							
Appearance	Off white to cream powder	Off white to cream powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder
Enumeration (CFU/g)	$1.34x10^{11}$	$3.40x10^{11}$	$1.32x10^{11}$	$4.00x10^{10}$	$6.40x10^{9}$	$3.20x10^{10}$	$1.70x10^{10}$
Total cell count (TCC/g)	$3.25x10^{11}$	$2.88x10^{11}$	$3.88x10^{11}$	$3.88x10^{11}$	$2.17x10^{11}$	$3.92x10^{11}$	$7.13x10^{11}$
Viability (%)	67.22	65.44	78.03	56.65	65.89	66.91	71.20
Moisture by Karl-Fischer (%)	5.06	4.74	3.34	3.87	3.53	2.30	3.66
Storage at 25°C							
Appearance	Off white to cream powder	Off white to cream powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder	Cream/light yellow powder
Enumeration (CFU/g)	$1.34x10^{11}$	$3.90x10^{11}$	$1.04x10^{11}$	$3.90x10^{10}$	$3.50x10^{9}$	$6.60x10^{10}$	$1.47x10^{10}$
Total cell count (TCC/g)	$3.25x10^{11}$	$2.58x10^{11}$	$5.85x10^{11}$	$2.92x10^{11}$	$3.54x10^{11}$	$4.21x10^{11}$	$3.58x10^{11}$
Viability (%)	67.22	60.60	60.15	57.96	65.16	69.54	60.87
Moisture by Karl-Fischer (%)	5.06	3.33	3.33	2.13	1.44	3.30	1.79

CFU = colony forming units; TCC = total cell count.

3. DIETARY EXPOSURE (21 CFR §170.235)

3.1 HISTORY OF USE IN FOODS

3.1.1 Uses as Starter Cultures

S. salivarius has a documented history of safe consumption in foods, with literature reports of its role as a starter culture in certain traditional fermented dairy products (*e.g.*, milks and cheeses in Europe, Africa, and Colombia) (Abdelgadir *et al.*, 2001; Callon *et al.*, 2004; Freire *et al.*, 2016; Jans *et al.*, 2017; Kadri *et al.*, 2021; Motato *et al.*, 2017; Obodai & Dodd, 2006; Ongol & Asano, 2009; Pešić-Mikulec & Jovanović, 2006; Van Hoorde *et al.*, 2008). The *Inventory of microbial food cultures with safety demonstration in fermented food products* compiled by the International Dairy Federation (IDF) and European Food and Feed Cultures Association (EFFCA) also includes *S. salivarius* (listed as "*S. salivarius* subsp. *salivarius*"), alongside the genetically related *S. thermophilus* (listed as "*S. salivarius* subsp. *thermophilus*") (Bourdichon *et al.*, 2018). Although *S. salivarius* has been used as a starter culture in fermented dairy products, its use in food production is less widespread than *S. thermophilus*, which is considered superior from a functionality perspective (Burton *et al.*, 2017; Marshall *et al.*, 1985).

S. thermophilus has been commonly used in the production of yogurt and cheese, perhaps since the domestication of animals and the origins of dairying practices (Burton *et al.*, 2017; Delorme, 2008). *S. thermophilus* is one of the most important industrial dairy starter cultures, being present in the millions of tons of yogurt and cheese that are commercially produced each year (Burton *et al.*, 2017; Delorme, 2008). Accordingly, the safety of *S. thermophilus* has been well established. *S. thermophilus* is included in European Food Safety Authority (EFSA) list of microorganisms with a qualified presumption of safety (QPS), with the generic qualification that strains should not harbor any acquired antimicrobial resistance genes to clinically relevant antimicrobials (EFSA, 2007; EFSA BIOHAZ Panel, 2020). In the U.S., the standards of identity for yogurt (21 CFR §131.200), lowfat yogurt (21 CFR §131.203), and nonfat yogurt (21 CFR §131.206) specifies *S. thermophilus* (with *Lactobacillus bulgaricus*) be used as the characterizing bacterial cultures that are used in the production of these foods. *S. thermophilus* is also listed as the microorganism to include in yogurt starter cultures in the Codex Alimentarius *Standard for Fermented Milks* (CXS 243-2003), alongside *Lactobacillus delbrueckii* subsp. *bulgaricus* or other *Lactobacillus* species (WHO/FAO, 2018). These starter organisms must be present at minimum levels of 10^7 CFU/g through to "*the date of minimum durability after the product has been stored under the storage conditions specified in the labelling*" (WHO/FAO, 2018). If other microorganisms are declared on the product label, they must be present at a minimum of 10^5 CFU/g.

In a review of published studies in which the content of live lactic acid bacteria (and other relevant bacteria) in commercially available fermented foods was assessed, it was reported that all the yogurts examined contained *S. thermophilus* and *L. delbruckii* subsp. *bulgaricus*, with levels of each ranging from $<10^4$ to 10^9 CFU per g or per mL (Rezac *et al.*, 2018). The samples were collected from the U.S., Australia, Spain, France, Norway, Greece, Argentina, and South Africa. The authors noted that assuming yogurt consumption is approximately 100 g/day, and if yogurt contained live microbes at levels of 10^8 CFU/g, this would correspond to intakes of 10^{10} CFU/day (Rezac *et al.*, 2018). Similarly, populations that

widely consume fermented foods have been estimated to ingest 10^8 to 10^{11} CFU/day of live microbes by other authors (Derrien & van Hylckama Vlieg, J. E., 2015; Lang *et al.*, 2014; Marco *et al.*, 2020).

3.1.2 Regulatory Status of *S. salivarius*

Other closely related strains of *S. salivarius*, namely *S. salivarius* K12 and M18 produced by BLIS Technologies Ltd., have been commercialized for use in foods and supplement-type products globally for many years. In the U.S., the FDA has "no questions" regarding the conclusions that *S. salivarius* K12 (GRN No. 591) and *S. salivarius* M18 (GRN No. 807) are GRAS for their intended conditions of use across a broad range of foods at levels providing a minimum of $1x10^9$ CFU/serving. The food categories include: baby, infant, and toddler foods (excluding infant formula); baked goods and baking mixes; beverage and beverage bases; breakfast cereals; cheeses; chewing gum; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; hard candy; milk, whole and skim; milk products; nuts and nut products; processed fruits and fruit juices; soft candy; sweet sauces, toppings, and syrups. It is anticipated that *S. salivarius* DB-B5 will be added to the similar food categories as those that have been concluded GRAS for the *S. salivarius* K12 and M18 strains in the U.S. (see Table 3.1.2-1).

The *S. salivarius* K12 and M18 strains also have regulatory clearance for use as a general food ingredient elsewhere. For instance, the Advisory Committee on Novel Foods at Food Standards Australia New Zealand (FSANZ) has determined that *S. salivarius* K12 and M18 are "not novel foods" (ACNF, 2020). In Canada, the Food Directorate at Health Canada has determined the use of *S. salivarius* K12 as a food ingredient is "not novel", on the basis that it has a history of safe use as a food[2].

Table 3.1.2-1 Examples of Potential Food Uses and Use Levels for *S. salivarius* DB-B5, based on the GRAS Uses for *S. salivarius* K12 and M18 in the U.S. (GRN No. 591 and 807)[a]

Food Category	Food Uses	Use Levels (CFU/serving)	Serving Size (g or mL)
Baby and Toddler Foods	Cereals, Baby Food	$1.0X10^9$	15 (dry, instant)[b] 110 (RTS)[b]
	Cookies, Crackers, and Puffs, Baby/Toddler Food	$1.0X10^9$	7[b]
	RTS Fruit-Based Baby/Toddler Food	$1.0X10^9$	60 (strained)[b] 110 (junior)[b] 125 (toddler)[b]
	Fruit Juices, Baby Food	$1.0X10^9$	125[b]
	RTS Dinners, Baby/Toddler Food	$1.0X10^9$	60 (strained)[b] 110 (junior)[b] 170 (toddler)[b]
	RTS Desserts, Baby Food	$1.0X10^9$	60 (strained) 110 (junior)
	RTF Vegetable-Based Baby/Toddler Food	$1.0X10^9$	60 (strained) 110 (junior) 70 (toddler)
Baked Goods and Baking Mixes	Cookies (chocolate coating)	$1.0X10^9$	20
Beverages and Beverage Bases	Meal Replacement powders (fortified, protein, and mineral replenish)	$1.0X10^9$	16 to 40

[2] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/requesting-novelty-determination/list-non-novel-determinations.html

Food Category	Food Uses	Use Levels (CFU/serving)	Serving Size (g or mL)
	Sports and Energy Drinks	$1.0X10^9$	250
	Water (Still or Mineral)	$1.0X10^9$	237
Breakfast Cereals	Breakfast Cereals	$1.0X10^9$	29
	Muesli and Dry Blended Cereals	$1.0X10^9$	85
Cheeses	Natural Cheeses	$1.0X10^9$	20 to 30
Chewing Gum	Chewing Gum	$1.0X10^9$	3
Dairy Product Analogs	Milk Substitutes	$1.0X10^9$	244
Frozen Dairy Desserts and Mixes	Frozen Yogurt	$1.0X10^9$	174
	Ice Cream	$1.0X10^9$	66
Gelatins, Puddings, and Fillings	Custards (pourable)	$1.0X10^9$	113
	Dessert Mixes (powder)	$1.0X10^9$	25
Grain Products and Pastas	Granola and Breakfast Bars	$1.0X10^9$	28
	Protein Bars	$1.0X10^9$	68
Hard Candy	Mint Candies	$1.0X10^9$	25
Milk, Whole and Skim	Milk (flavored, pasteurized)	$1.0X10^9$	244
	Milk (fresh)	$1.0X10^9$	244
	Milk Powder (skim or whole)	$1.0X10^9$	23 to 32
Milk Products	Cream (pasteurized)	$1.0X10^9$	244
	Cultured Milk Products	$1.0X10^9$	180
	Dairy Desserts	$1.0X10^9$	100 to 180
	Milkshake Mixes (powder)	$1.0X10^9$	21
	Yogurt	$1.0X10^9$	227
	Yogurt Drinks	$1.0X10^9$	244
Nuts and Nut Products	Peanut Butter	$1.0X10^9$	32
Processed Fruits and Fruit Juices	Fruit-Flavored Beverages (powder)	$1.0X10^9$	18
	Fruit Juices	$1.0X10^9$	263
	Fruit Juice Drinks	$1.0X10^9$	209
Soft Candy	Chewable Lozenges	$1.0X10^9$	3
	Chocolate Bars	$1.0X10^9$	44
	Soft Gel and Rapid Melt Technologies	$1.0X10^9$	2
Sweet Sauces, Toppings, and Syrups	Cinnamon, Nutmeg, and Chocolate Sprinkle	$1.0X10^9$	4[b]
	Sugar and Sweetener Sprinkle	$0.5x10^8$	4[b]

CFU = colony forming units; RTF = ready to feed; RTS = ready to serve

[a] Reproduced from Table 1.3-1 of the GRAS notice for *S. salivarius* M18 (GRN No. 807), which is intended for the same food uses as those for the *S. salivarius* K12 strain (GRN No. 591). The serving sizes indicated in this table were provided by BLIS Technologies, unless otherwise indicated by footnote b.

[b] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in 21 CFR §101.12.

3.2 Estimated Daily Intake of *S. salivarius* DB-B5

Intake modelling was used to derive the estimated intake of *S. salivarius* K12 from its intended uses in the U.S., the results of which have been previously described in GRN No. 591 and were incorporated by reference for *S. salivarius* M18 in GRN No. 807. Using food consumption data available in the 2003-2004 and 2005-2006 National Health and Nutrition Examination Surveys (NHANES), the 90th percentile all-user estimated intake from the intended food uses of *S. salivarius* K12 and M18 was determined to be in the ranges of approximately $2x10^{10}$ CFU/person/day (see Table 3.2-1).

Table 3.2-1 Estimated Daily Intake of *S. salivarius* K12 and M18 from their Intended Uses in the U.S. (2003-2004, 2005-2006 NHANES Data)

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

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

[a] Reproduced from the GRAS notice for *S. salivarius* M18 (GRN No. 807). The estimated daily intakes for *S. salivarius* M18 are identical to those estimated for *S. salivarius* K12 in GRN No. 591.

The estimated intake of *S. salivarius* DB-B5 from its intended uses as a general ingredient in conventional foods is anticipated to be within the ranges of those previously estimated for *S. salivarius* K12 and M18. The target use level of *S. salivarius* DB-B5 in foods are the same as the use levels for *S. salivarius* K12 and M18 (*i.e.*, $1x10^9$ CFU/serving), which reflects the typical inclusion rates for other live microbial cultures employed in the food industry (Champagne *et al.*, 2005). Although the intended uses of *S. salivarius* DB-B5 are largely substitutional for the uses of *S. salivarius* K12 and M18, it is possible that *S. salivarius* DB-B5 may be added to food products that were not covered under the scope of the exposure assessment described in GRN No. 591 and 807. Nonetheless, as stated in GRN No. 807: *"It is expected that food uses of S. salivarius M18 would generally be substitutional to food uses of S. salivarius K12; however, as M18 is not intended to serve as a replacement for K12, some additive consumption may occur on occasion. Given the logarithmic nature of microorganism counts, even a doubling of the intake estimates described below in Table 3.2-1 would remain with the 10^{10} CFU count range."*

It should also be noted that the intake levels derived previously for *S. salivarius* K12 and M18 are greatly overestimated to begin with. As discussed in GRN No. 591 and 807, the methodologies employed yield estimates that would occur under the 'worst-case' scenario, due to several conservative assumptions made in their derivation. Moreover, to reach the level of intakes derived for the intended food uses of *S. salivarius* K12 and M18 (*i.e.*, approximately $2x10^{10}$ CFU/person/day), approximately 20 servings of foods containing *S. salivarius* DB-B5 would need to be consumed daily if the strain is added at $1x10^9$ CFU/serving. It is highly unlikely that 20 servings of foods containing *S. salivarius* DB-B5 would be consumed, as this reflects the amount of **all** foods that would be typically consumed in a day (Basiotis *et al.*, 2000). In the GRAS notices of other live microbial strains that were similarly intended for use as food ingredients (*e.g.*, GRN No. 377; GRN No. 601; GRN No. 736; GRN No. 831; GRN No. 847; GRN No. 856; GRN No. 953), an extremely conservative estimation of intake was derived on the basis that an average individual consumes approximately 20 servings/day of all foods combined, and assuming the strain of interest would be present in all those foods at the specified CFU per serving. As a more realistic approach, even if it was assumed that only half of the foods consumed will contain the strain of interest (*i.e.*, 10 servings per day), this was still viewed as a conservative approach in the derivation of exposure. In fact, consumption of just 5 servings of foods containing an added live microbial strain was considered an "extreme" case of high intake (*e.g.*, GRN No. 905).

4. Self-Limiting Levels of Use (21 CFR §170.240)

The addition of *S. salivarius* DB-B5 is limited to foods that will sustain the viability of the strain through the shelf-life of the food product. The inclusion rate of *S. salivarius* DB-B5 to foods is not self-limiting, in that there are no alterations to palatability, and it does not become technologically impractical above a certain addition level. Nonetheless, the addition level of *S. salivarius* DB-B5 to foods are unlikely to exceed those indicated in Section 1.4 (*i.e.*, target of $1x10^9$ CFU/serving) as it would become cost-prohibitive to do so.

5. Experience Based on Common Use in Food Before 1958 (21 CFR §170.245)

Not applicable. The GRAS status of *S. salivarius* DB-B5 for its intended uses in foods is established through scientific procedures.

6. Safety Narrative (21 CFR §170.250)

6.1 Rationale

S. salivarius is a commonly occurring human commensal organism; it is predominant member of the oral microbiota starting from birth and is also located at other sites such as the oropharynx, skin, and intestinal and genitourinary tract (Public Health Agency of Canada, 2018; Wescombe *et al.*, 2012). A high degree of genetic similarity exists between *S. salivarius* and *S. thermophilus*, which has an established history of safe use as an industrial starter culture. Other strains of *S. salivarius*, such as *S. salivarius* K12 and M18, are GRAS for use as food ingredients in the U.S. (GRN No. 591, 807).

Dose Biosystems has extensively characterized the *S. salivarius* DB-B5 strain. Taxonomical identity was confirmed by whole genome sequencing, and bioinformatic analyses demonstrates the absence of transmissible antibiotic resistance genes or virulence factors in the genome, as described further below in Section 6.5. Phenotypic testing further showed *S. salivarius* DB-B5 to be susceptible to clinically relevant antibiotics (see Section 6.6). Moreover, *S. salivarius* DB-B5 has been safely consumed by humans without adverse effects in 2 clinical studies (see Section 6.4.1). In addition to the data that have been gathered for *S. salivarius* DB-B5, its safety can be corroborated by the studies that have been conducted on *S. salivarius* K12 and M18, which have been described in detail in the GRAS notices for these strains (GRN No. 591 and 807) and are incorporated by reference herein (Section 6.4.2). A comprehensive search of the literature was also conducted by Dose Biosystems to identify additional studies pertinent to the safety of *S. salivarius* DB-B5 that have been published through to May 2021. A primary search was conducted with the Scopus database, using the search terms listed Table 6.1-1. Secondary searches were also conducted with PubMed and Google Scholar.

The data and information to support the safety of *S. salivarius* DB-B5 for its intended conditions of use as a food ingredient are described in the sections that follow. All the pivotal data and information used to establish the safety of *S. salivarius* DB-B5 under its intended conditions of use in foods have been

published and are available in the public domain. The safety of the species for use in food has also been the subject of multiple systematic and comprehensive reviews by qualified experts (see Section 3.1 above, and as incorporated by reference from GRN No. 807).

Table 6.1-1 Search Terms Used to Identify Literature Pertinent to the Safety of *S. salivarius* in Scopus

Parameter[a]	String
Species	"Streptococcus salivarius" OR "S salivarius"
Outcomes	
Antimicrobial Resistance	"antimicrobial AND resistan*" OR "antibiotic AND resistan*" OR "antimicrobial AND susceptibil*"
Infection/Bacteremia/Fungemia/Sepsis	infection* OR abscess* OR sepsis* OR septic* OR bacteremia OR bacteraemia OR toxin*
Clinical Study/Morbidities/Mortalities	clinical* OR trial* OR supplement* OR death* OR morbidit* OR mortalit* OR disease* OR illness*
Disease Risk	opportunistic OR virulen*

[a] Article title, abstract, and keywords were searched using the terms listed in this table. The search terms were adapted with minor modifications from those used by EFSA for the maintenance and update of the list of QPS-recommended biological agent, specifically those applied to *S. thermophilus* (https://doi.org/10.5281/zenodo.3607193). No date restrictions were placed on the search.

6.2 Metabolic Fate

6.2.1 Occurrence of *S. salivarius* as a Human Commensal

The body comprise a complex community of resident microbes that have coevolved and coexisted with humans in a mostly harmonious symbiotic relationship (Kilian *et al.*, 2016). The warm, moist, and nutrient-rich environment of the oral and nasopharyngeal cavity provides an ideal environment in which microorganisms can flourish (Abranches *et al.*, 2018; Deo & Deshmukh, 2019; Kilian *et al.*, 2016). In fact, the oral cavity has one of the largest and most diverse bacterial population in the body (Kilian *et al.*, 2016). Once established, the oral microbiota is maintained by a combination of host- and microbe-derived factors; in healthy individuals, the oral biofilm is dominated by commensal bacteria that helps to maintain the homeostasis integral to health (Abranches *et al.*, 2018; Kilian *et al.*, 2016).

Streptococci are commensal organisms that are ubiquitously present throughout the human body; they are widespread in most, if not all, mucosal surfaces, especially within the oral cavity and upper respiratory tract where they are known to be the predominant species (Abranches *et al.*, 2018; Nobbs *et al.*, 2009; Ruiz *et al.*, 2019). Streptococci are amongst the first organisms to colonize the human oral cavity from birth, and *S. salivarius* is recognized as one of these pioneer species, being frequently detected in the oral cavity of infants (Wescombe *et al.*, 2012; Xiao *et al.*, 2020). *S. salivarius* remains a predominant member of the commensal oral microbiota throughout life, persisting especially in the tongue dorsum, and it is also dominant species in the pharyngeal mucosa (Horz *et al.*, 2007; Human Microbiome Project Consortium, 2012; Wescombe *et al.*, 2009). Additionally, *S. salivarius* has been identified as a commensal organism at other sites in the body such as the skin, the gastrointestinal and genitourinary tracts, as well as in breastmilk (Delorme *et al.*, 2015; Public Health Agency of Canada, 2018).

The levels of *S. salivarius* in saliva samples taken from children and adults have been reported to range from 10^7 to 10^8 CFU per mL (Amoroso *et al.*, 2003; Burton *et al.*, 2010; Loesche *et al.*, 1995). Considering that the total volume of saliva produced per day is approximately 500 mL in children (Watanabe *et al.*, 1995) and up to 1.5 L for adults (Hall, 2011; Humphrey & Williamson, 2001; Iorgulescu, 2009), humans are estimated to ingest approximately $5x10^9$ to $1.5x10^{11}$ CFU/day of commensal *S. salivarius*. Thus, exposure to *S. salivarius* occurs daily in all humans across all age groups. Moreover, the transfer of commensal microbial strains is expected to occur between individuals through normal social interactions (*e.g.*, kissing, sharing of foods and utensils) (Han *et al.*, 2016; Hesselmar *et al.*, 2013; Kort *et al.*, 2014).

6.2.2 Colonization and Metabolic Fate of *S. salivarius* DB-B5

Permanent lifelong colonization by ingested microorganisms is thought to be rare (WHO/FAO, 2009). In a recent review of the literature, it was noted that supplementation with live bacteria cultures is likely to increase the fecal count of the specific bacterial strains that were administered to healthy adults, though the changes in the gut microbiota were temporary and returned to pre-treatment levels within 1 to 3 weeks following cessation of the supplementation (Khalesi *et al.*, 2018). Similarly, clinical studies have suggested that colonization by *S. salivarius* K12 in the oral cavity is transient, with levels declining once supplementation is stopped (Burton *et al.*, 2011; Horz *et al.*, 2007; Sarlin *et al.*, 2021). Moreover, colonization by *S. salivarius* K12 and M18 may be subject to inter-individual variability, with colonization being detected in only a subset of the participants studied (Burton, Drummond *et al.*, 2013; Power *et al.*, 2008).

Evidence of colonization by *S. salivarius* DB-B5 in the oral cavity has been observed in a clinical trial (NCT04473404). Additional details of this study are described further in Section 6.4.1. Briefly, healthy adults were randomized to receive sachets providing *S. salivarius* DB-B5 at $2x10^9$ CFU/day (n=15), *S. salivarius* DB-B5 at $1x10^{10}$ CFU per day (n=16), or a placebo control (n=16) for 4 weeks. The sachets were consumed twice daily. On each occasion, the participants dissolved 1 sachet in approximately 4 ounces of bottled water, and then sipped the test product until it is completely consumed. At the end of study, DB-B5 was detected in both saliva and tongue scrapings of treatment participants using quantitative PCR.

Since *S. salivarius* DB-B5 is expected to exert their effects primarily within the oral cavity, the strain has not been assessed for acid or bile salt resistance. Nonetheless, it has been reported that other isolates of *S. salivarius* obtained from human breastmilk did not survive when they were tested under *in vitro* conditions simulating gastric environment (pH 2.0 in the presence of pepsin) (Damaceno *et al.*, 2017). The *S. salivarius* isolates were resistant to degradation by bile salts (Oxgall at 2%), though the authors noted that *"a bacterial isolate that could not resist an initial acidic stress would have little chance of surviving throughout the rest of the gastrointestinal tract"* (Damaceno *et al.*, 2017). Similarly, experimentation in rats administered a mixture of live microbial strains (Biorestore™ containing $3.9x10^9$ CFU *L. acidophilus* LA742, $2.3x10^{10}$ CFU *L. rhamnosus* L2H, $8.0x10^9$ CFU *B. lactis* HN019, and $1.1x10^{10}$ CFU *S. salivarius* K12) by gavage twice daily for 3 days suggest that *S. salivarius* K12 does not persist in the gastrointestinal tract (Lee *et al.*, 2009). *S. salivarius* was also not detected in the gastrointestinal tract in another study where a different mixture of live microbial strains was administered to rats by gavage twice daily for 3 days (*i.e.*, *L. acidophilus* L10, *L. rhamnosus* 67B, *B. lactis* LAFTI® B94, and *S. salivarius*

K12 in equal proportions for a total of approximately $1x10^{10}$ CFU/ml) (Krittaphol *et al.*, 2011). With respect to these results, it has been stated in the GRAS notice for *S. salivarius* M18 (GRN No. 807) that:

> *"As discussed in GRN 591, the species S. salivarius is specific to humans and therefore findings in rodent studies are of unclear relevance to the in vivo situation in humans. Consumption of S. salivarius M18 in the diet is not expected to affect the microbiota composition of the gut, particularly given that consumption of indigenous strains of S. salivarius within saliva occurs in all individuals on a continual basis. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the cellular components (proteins, lipids, carbohydrates) used as a source of nutrients. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the feces."*

6.3 Preclinical Studies

It has been recognized that traditional preclinical toxicological tests have limitations with respect to their relevance in the safety evaluation of live microbial species for human consumption. For instance, it was noted in the GRAS notice for *S. salivarius* K12 (GRN No. 591) and reiterated again in the subsequent GRAS notice for *S. salivarius* M18 (GRN No. 807) that: *"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)."* Moreover, in a review published a panel of experts who had convened at the 7th Annual Conference of the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Shane *et al.*, 2010), it was stated that: *"For most chemical substances, most of the burden of evaluating safety falls on tests performed on well-understood animal models. For the safety-related endpoints important in the assessment of probiotics, validated animal models do not exist and, as a result, the determination of safety rests primarily on human studies."*

Accordingly, preclinical toxicology studies have not been conducted with *S. salivarius* DB-B5, though clinical studies provide support that the strain is safe for human consumption (see Section 6.4.1). It is also worth noting that traditional toxicological studies have been conducted for the *S. salivarius* K12 strain. As detailed in GRN No. 591, *S. salivarius* K12 was not mutagenic when tested using a bacterial reverse mutation assay (Burton *et al.*, 2010). *S. salivarius* K12 also did not produce any evidence of toxicity when it was evaluated in an acute oral toxicity study and a 28-day oral toxicity study in rats (Burton *et al.*, 2010). A summary of these studies is presented in Table 6.3-1 below; the lack of adverse effects observed in these studies provides further corroborative evidence of the safety of *S. salivarius*. In addition, several studies have been conducted with *S. salivarius* K12 and other strains in various mechanistic animal models. One study has also been conducted to evaluate the effects of a pig-derived *S. salivarius* NBRC13956 strain (both alone or as a multi-strain preparation) on growth performance and blood parameters in piglets; however, details of the study designs and results were poorly reported, with unclear information provided on the doses administered, species used, and methodologies for the statistical analyses (Dlamini *et al.*, 2017). Although these studies generally hold limited value for the safety assessment of *S. salivarius* DB-B5, they are nonetheless summarized in Table 6.3-1 for completeness. Overall, the results of these animal studies do not raise any concerns with regards to the safety of *S. salivarius* DB-B5 as a food ingredient.

Table 6.3-1 Summary of Published Animal Studies Conducted with *S. salivarius*

Reference	Animals	Study Duration	Route of Administration	Strain Tested	Administration Levels	Safety-Related Outcomes
Toxicology Studies Conducted with *S. salivarius* K12						
(Burton *et al.*, 2010)	Sprague-Dawley rats (59 total)[a]	Single bolus dose administered	Gavage	*S. salivarius* K12	**Test 1**: 1.25x10^{8} CFU/rat (7.5 mg/kg bw) **Test 2**: 1.67x10^{9} CFU/rat (100 mg/kg bw) **Test 3**: 8.00x10^{10} CFU/rat (5,000 mg/kg bw) **Control 1**: lyoprotectant **Control 2**: saline	• No abnormal findings were detected in any of the tested animals, with no effects on daily health scores or food consumption. • No evidence of septicemia or acute bacterial infection of the heart valves and pharyngeal tissues at 48-hours. No infection or tissue abnormalities at Day 14. • *S. salivarius* K12 does not have an acute toxic effect when orally administered.
	Sprague-Dawley rats (20/sex/group)	28 days	Dietary	*S. salivarius* K12	**Test 1**: 7.5 mg/kg bw/d **Test 2**: 100 mg/kg bw/d **Test 3**: 5,000 mg/kg bw/d **Control**: lyoprotectant	• No adverse effects on general clinical signs, ophthalmologic evaluations, organ weights, or gross pathology. • No toxicologically relevant, treatment-related changes were observed in body weight; in hematology, serum biochemistry, and urinalysis parameters; and form histopathology examination.
Other Animal Studies Conducted with *S. salivarius* Strains						
(Dlamini *et al.*, 2017) ***New study since GRN No. 807***	Weaned piglets (9/group, sex NR) Included a commercial breed (large white x landrace) and a South African Windsnyer breed	30 days	Dietary	*S. salivarius* NBRC13956, alone or with other live microbial species	**Control (NC)**: diet only **Control (PC)**: diet with antibiotic (lyncospectin) **Test (P1)**: diet with *L. reuteri* ZJ625 **Test (P2)**: diet with *S. salivarius* NBRC13956 **Test (P3)**: diet with *S. salivarius* NBRC13956, *L. reuteri* ZJ625, *L. reuteri* VB4, and *L. salivarius* ZJ614 Dietary concentrations were reported as CFU/mL, even though the diet appears to be in pellet form (*e.g.*, avg 2.9x10^{10} CFU/mL	• NSD in feed intake between groups. • Average daily gains and feed conversion ratio were SS ↑ in the P3 group compared to other groups. • NSD in total serum protein, cholesterol, and glucose between groups. • Serum albumin and globulin were SS ↓ in P1, P2, P3 and PC when compared to NC. • NSD in hematology parameters between P2 vs. NC, except SS ↓ in segmented neutrophils in P2 (as well as PC, P1, and P3) when compared to NC. • ↑ IgG serum concentrations in P1, P2, and P3 compared to the controls (PC and NC; unclear if difference is SS) • Overall, authors concluded that "*probiotics have beneficial effects on growth*

Reference	Animals	Study Duration	Route of Administration	Strain Tested	Administration Levels	Safety-Related Outcomes
					for *S. salivarius* NBRC13956). Dosage levels on a CFU/day basis were not provided.	*performances, blood parameters, and IgG stimulation of weaned piglets."*
(Hamada *et al.*, 1978)	Sprague-Dawley rats (sex and number NR)	Unclear; experimental period was stated as 85 to 122 days	"Inoculation" and in drinking water	*S. salivarius* HT9R, HT3R	Inoculation with 10^{12} CFU on Day 5, followed by 10^{10} CFU/ml in drinking water	• *S. salivarius* strains were not cariogenic.
(Ishijima *et al.*, 2012)	Female ICR mice (7 to 15/group)	5 time-points: at 24h, 3h before, and 3h, 24h, 27 h after *C. albicans* inoculation	Oral (round-top needle used to apply treatment throughout the mouth)	*S. salivarius* K12	**Test:** 50 µL solution applied at 3 levels of *S. salivarius* K12: • $7.5x10^8$ CFU/ml • $1.5x10^9$ CFU/ml • $3x10^9$ CFU/mL **Control 1**: water **Control 2**: fluconazole	• Oral treatment with *S. salivarius* K12 protected the mice from severe candidiasis.
(Lee *et al.*, 2012)	Male Wistar rats (5/group in *ex vivo* study)	3 days	Gavage	*S. salivarius* K12 with other live microbial species	**Test**: 2 g/day of BLIS BioRestore™ containing *S. salivarius* K12 ($1×10^8$ CFU/g), *L. acidophilus* LAFTI® L10 ($4×10^8$ CFU/g), *B. lactis* LAFTI® B94 ($4×10^8$ CFU/g) **Control**: excipients of BLIS BioRestore™	• BLIS BioRestore™ increased azoreductase activity in the colon content.
(Patras *et al.*, 2015)	Female CD1 mice (7 to 20/group)	5 days post inoculation with *S. agalactiae*	Vaginal inoculation	*S. salivarius* K12	**Test**: $1x10^8$ CFU/dose **Control**: PBS only	• *S. salivarius* K12 significantly reduced vaginal colonization with *S. agalactiae* (group B streptococcus)
(Tanzer *et al.*, 1985)	Osborne Mendel rats (sex NR; 12 to 13/group)	Orally inoculated with *S. salivarius* on 3 occasions at 8 days after inoculation with *S. mutans*		*S. salivarius* TOVE-R	$6x10^8$ cells of *S. salivarius* TOVE-R per dose	• NSD in body weight gain.

Reference	Animals	Study Duration	Route of Administration	Strain Tested	Administration Levels	Safety-Related Outcomes
	Osborne Mendel rats (sex NR; 9/group)	Orally inoculated with *S. salivarius* on 3 occasions at 7 days after inoculation with *S. sobrinus*		*S. salivarius* TOVE-R	$6x10^8$ cells of *S. salivarius* TOVE-R per dose	• NSD in body weight gain.
(Riane *et al.*, 2020) ***New study since GRN No. 807***	Female Wistar rats (5/group)	7 days	Gavage	*S. salivarius* St.sa	**Test 1**: 10^9 CFU/day **Test 2**: single dose of diclofenac on day 7 **Test 3**: 10^9 CFU/day plus diclofenac on day 7 **Control**: saline	• No mortality observed in any groups. • Administration of *S. salivarius* St. sa did not adversely affect biomarkers of liver function (ALP, AST, ALT). • Levels of malondialdehyde and glutathione, and antioxidant enzymes (superoxide dismutase, catalase), in rat livers were similar between *S. salivarius* St. sa group and controls.

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate transferase; avg = average; bw = body weight; CFU = colony forming units; d = day; NR = not reported; NSD = no significant difference; PBS = phosphate-buffered saline.

[a] From GRN No. 807, it appears there were 6/sex/group in the test groups and control 1 (receiving lyoprotectant), and 3/sex/group in control 2 (receiving saline). One additional male rat was included in each group for termination at 48 hours. The remaining animals were monitored twice daily for 14 days following administration of the test articles.

6.4 Clinical Data

6.4.1 Studies Conducted with *S. salivarius* DB-B5

Two independent randomized, double-blind, placebo-controlled clinical studies have been conducted with *S. salivarius* DB-B5. In one study (NCT04492631), the primary objective was to specifically evaluate the safety and gastrointestinal tolerability of *S. salivarius* DB-B5 in healthy adults (Li *et al.*, 2021). Individuals between the ages of 18 to 65 years old with a normal body mass index (BMI) of 18.5 to 35 kg/m^2 were included in the study. The participants were randomized to receive either *S. salivarius* DB-B5 at $1x10^{10}$ CFU per day (n=32) or a placebo control (n=32) for 4 weeks. The test products were provided as single-use sachets that contained *S. salivarius* DB-B5 with a mannitol carrier, or placebo sachets that contained mannitol only. Each day after breakfast, the participants dissolved 1 sachet in approximately 4 ounces of bottled water, and then sipped the test product until it is completely consumed. The test powder with *S. salivarius* DB-B5 were packaged in the same manner as the control powder. A fasting blood and urine sample was collected at screening (Day -21 to -3), baseline (Day -1), and end-of-study visit (Day 29 (+3)) for the analysis of standard laboratory parameters (*i.e.*, hematology, clinical chemistry, and urinalysis). To assess tolerability, the participants completed the Gastrointestinal Symptom Rating Scale (GSRS) at the screening, baseline and end-of-study visits.

Out of the 64 participants who were randomized, there were 4 participants who did not complete the 28-day intervention. One participant in the *S. salivarius* DB-B5 group discontinued from the study on Day 1 after changing their mind about participation. The remaining 3 participants were from the placebo group; 1 was lost due to follow-up, 1 was discontinued due to elevated eosinophils at the baseline blood sample, and 1 experienced mild urticaria that was considered possibly related to the study product by the investigator. A high degree of compliance was observed in this study; all 31 participants (100%) in the *S. salivarius* DB-B5 group, and 26 participants (83.9%) in the placebo group, consumed all 28 doses of their allocated test products. In the *S. salivarius* DB-B5 group, 2 participants reported a total of 5 adverse events (AEs) throughout the study that were considered "possibly related" to the interventions. One participant reported 2 separate occasions of bloating, and 1 participant reported 3 separate occurrences of flatulence. All these events were mild in nature and resolved on their own. In the placebo group, 4 participants reported a total of 10 AEs (bloating, constipation; headache, urticaria; loose stools, stomach cramps; myalgia, rhinorrhea, sinus headache).

The low incidence of gastrointestinal-related AEs is in agreement with the low scores reported from the GSRS. The mean scores for each of the 15-items assessed in the GSRS were all <2 ("minor discomfort"). No statistically significant differences were observed in any of the GSRS symptoms over the course of the study in both groups, and there were no statistically significant differences in the change of GSRS scores between the *S. salivarius* DB-B5 and placebo groups. There were no statistically significant differences in the vital signs (*i.e.*, systolic and diastolic blood pressure, heart rate, respiratory rate, and body temperature) or laboratory analyses (*i.e.*, hematology, clinical chemistry, urinalysis) between intervention groups or visits. All laboratory values were either within normal ranges or were deemed to be non-clinically significant by the study physician. Overall, this study demonstrates that consumption of *S. salivarius* DB-B5 is safe and well-tolerated.

In another study (NCT04473404), the effects of *S. salivarius* DB-B5 supplementation on oral health was investigated in adults (age 18 to 65) with good general health and good oral health. A total of 48 individuals were randomized into the study, though 1 person withdrew consent at the baseline visit due to scheduling conflicts. The participants in this study received *S. salivarius* DB-B5 at $2x10^9$ CFU/day (n=15), *S. salivarius* DB-B5 at $1x10^{10}$ CFU per day (n=16), or the placebo control (n=16) for 4 weeks. All of these participants completed the study. The test products were provided as single-use sachets that contained only mannitol as a placebo control, or sachets that contained *S. salivarius* DB-B5 with a mannitol carrier at either $1x10^9$ CFU/sachet or $5x10^9$ CFU/sachet. The participants consumed 2 sachets daily, once in the morning after breakfast and once in the evening after dinner. On each occasion, the participants dissolved 1 sachet in approximately 4 ounces of bottled water, and then sipped the test product until completely consumed. There were no observed and/or reported evidence of any hard or soft tissue damage upon examination of the oral cavity by the study dentist. One AE was reported in the study by one participant (cheek bite), which was deemed not related to the intervention product. There were no serious AEs observed in this study.

6.4.2 Studies Conducted with Other *S. salivarius* Strains

Various clinical studies have also been conducted with other *S. salivarius* strains, as summarized in Table 6.4.2-1. These studies have been described in the previous GRAS notices for *S. salivarius* K12 (GRN No. 591) and *S. salivarius* M18 (GRN No. 807). Dose Biosystems also conducted a search of the literature to identify additional publications that have become available since the filing of these GRAS notices. These studies are marked as such in Table 6.4.2-1.

One randomized, double-blind, placebo-controlled clinical trial has been conducted to specifically evaluate the safety of *S. salivarius* K12 (Burton *et al.*, 2011). In this study, administration of *S. salivarius* K12 in powdered sachets at levels of $1.1x10^{10}$ CFU/day for 28 days was demonstrated to be well tolerated. The adverse events that were reported in the *S. salivarius* K12 group were either not considered related to the intervention, or were otherwise gastrointestinal events (dyspepsia, flatulence) that were mild in nature. No clinically significant differences were observed in the hematology, clinical chemistry, and urinalysis parameters between the *S. salivarius* K12 and placebo control group.

The remainder of the studies identified were designed to evaluate the effect of *S. salivarius* K12 and M18 on various health outcomes among pediatric and adult populations. Although they were not designed to investigate safety-related endpoints as the primary outcome, the absence of AEs across these studies further supports that the consumption of *S. salivarius* strains is safe and well-tolerated when consumed daily over prolonged durations, at levels ranging 10^9 to 10^{10} CFU/day. Additionally, clinical studies have been published in which *S. salivarius* 24SMB, in combination with *S. oralis* 89a, was administered to children and adults in the form of a nasal spray (*e.g.*, Bellussi *et al.*, 2018; Cantarutti *et al.*, 2020; De Grandi *et al.*, 2019a; De Grandi *et al.*, 2019b; La Mantia *et al.*, 2017; Manti *et al.*, 2020; Marchisio *et al.*, 2015; Passali *et al.*, 2019; Santagati *et al.*, 2015; Tarantino *et al.*, 2018; Tarantino *et al.*, 2019) or an oral spray (Andaloro *et al.*, 2019; Tarantino *et al.*, 2020). Although the route of administration for *S. salivarius* 24SMB (*i.e.*, nasal or oral spray) is not reflective of the exposure that would occur from the intended uses of *S. salivarius* DB-B5 as a food ingredient, the lack of treatment-related adverse effects in these studies also provide corroborative evidence for the safety of *S. salivarius*.

Table 6.4.2-1 Summary of Clinical Studies Conducted with *S. salivarius* DB-B5 and Other *S. salivarius* Strains

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
Studies Conducted with *S. salivarius* DB-B5						
(Li *et al.*, 2021) NCT04492631	To investigate the safety and tolerability of *S. salivarius* DB-B5.	Randomized, double-blind, placebo controlled, parallel	Healthy adults (M & F; age 18 to 65 y) $n_I = 64$ $n_T = 60$	**Test**: $1x10^{10}$ CFU/sachet **Control**: matched placebo Sachets containing *S. salivarius* DB-B5 or control were dissolved in 4 oz. of water and consumed once daily in the morning ($1x10^{10}$ CFU/day).	28 days	• 1 participant in the test group changed their mind on Day 1 and withdrew from the study. 3 participants (control group) were discontinued from the study due to AEs (n=2) or were lost to follow-up (n=1). • 2 participants in the test group reported 5 GI-related AEs, which were all mild and resolved on their own. • NSD in GSRS scores between groups. • NSD in vital signs or laboratory analyses (hematology, clinical chemistry, urinalysis) between groups. • Consumption of *S. salivarius* DB-B5 was considered safe and well tolerated.
Unpublished (NCT04473404)	To investigate the effects of *S. salivarius* DB-B5 on salivary, plaque, and tongue bacteria levels, and on oral malodor.	Randomized, double-blind, placebo controlled, parallel	Healthy adults (M & F; age 18 to 65 y) $n_I = 48$ $n_T = 47$	**Test 1**: $1x10^9$ CFU/sachet **Test 2**: $5x10^9$ CFU/sachet **Control**: matched placebo Sachets containing *S. salivarius* DB-B5 or control were dissolved in 4 oz. of water and consumed twice each day (up to $1x10^{10}$ CFU/day).	28 days	• 1 participant withdrew from the study due to scheduling conflict. • 1 AE was reported in 1 participant (cheek bite), which was not product related.

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
Studies Conducted with *S. salivarius* K12						
(Burton *et al.*, 2006)	To evaluate the effect of *S. salivarius* K12 supplementation on the composition of the oral microbiota	Open-label, single-arm	Healthy adults (M & F; mean age 19 y) n = 14	**Test**: ca. $1x10^9$ CFU/lozenge Lozenges were consumed at 2h intervals for 8 hours each day (*i.e.*, $4x10^9$ CFU/day).	3 days	• No adverse symptoms were reported by any of the participants.
(Burton, Chilcott *et al.*, 2006)	To evaluate the effect of *S. salivarius* K12 on oral malodor and the oral microbiota composition.	Open-label observational	Healthy adults (M & F; age 19 to 69 y) with VSC scores higher than 200 ppm at baseline n = 23 (initial 3-day study)	**Test**: $>1x10^9$ CFU/lozenge **Control**: placebo lozenge 3-day regimen of CHX rinsing, followed by intake of lozenges (test, control) at 2h intervals over 8h for 3 days ($>4x10^9$ CFU/day). Subsequently, subjects in the test group (n=13) took the lozenge twice daily (morning & night) for 2 weeks. Two of these subjects continued to take 2 lozenges/day for 28 days ($>2x10^9$ CFU/day).	3 days, 2 weeks (test group only), 28 days (2 subjects in test group only)	• Study authors did not report whether any AEs were observed by the participants.
(Burton *et al.*, 2010)	To examine the extent of colonization in the oral cavity after *S. salivarius* K12 administration.	Randomized, parallel Blinding NR	Healthy adults (M & F; mean age 19 y) n_i = 100 n_f = NR	**Test 1**: $1.5x10^9$ CFU/lozenge **Test 2**: $1.1x10^8$ CFU/lozenge **Test 3**: $2x10^7$ CFU/lozenge **Test 4**: $1x10^6$ CFU/lozenge **Test 5**: $7.5x10^4$ CFU/lozenge One lozenge was consumed daily (up to $1.5x10^9$ CFU/day).	14 days	• No adverse reactions were reported by the participants.
(Burton *et al.*, 2011)	To evaluate the safety and tolerability of *S. salivarius* K12.	Randomized, double-blind, placebo controlled, parallel	Healthy adults (M & F) age 20 to 60 y n_i = 56 n_f = 53	**Test**: $1.1x10^{10}$ CFU/sachet **Control**: matched placebo Sachets were dissolved in 4 oz. of water and consumed each day at breakfast ($1.1x10^{10}$ CFU/day).	28 days	• NSD in oral health endpoints assessed using a 10-point VAS • NSD in GI symptoms assessed using a 10-point VAS • No serious AEs occurred in either intervention

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
						groups. Proportion of participants reporting at least one AE in the test group (29.6%) is similar to control (37.9%), and the proportion of AEs considered possibly attributable to the treatments was low. • NSD in changes in vital signs from end of treatment and baseline between groups. • NSD in hematology or clinical chemistry parameters. • SS ↑ in specific gravity of the urine in the placebo group compared to the test group, but values remained within normal limits. NSD in other urinalysis parameters.
(Cohen *et al.*, 2013)	To determine whether follow-up formula supplemented with a multi-strain mixture and prebiotic reduces the incidence of acute otitis media episodes in children	Randomized, double-blind, placebo controlled, parallel	Infants (M & F; 7 to 13 months) with high risk of acute otitis media n_I = 224 n_f = 202 by 2-month visit, 166 by 12-month visit	**Test**: follow-up formula containing proB (*S. thermophilus* NCC 2496, *S. salivarius* DSM 13084 [K12], *L. rhamnosus* LPR CGMCC 1.3724) and preB [Raftilose/Raftiline]) **Control**: follow-up formula only Formula contained $2.5x10^7$ CFU/g *S. salivarius* ($1x10^9$ to $2x10^9$ CFU/day according to GRN 807).	12 months	• Both the test and control formulas were considered well-tolerated by the study authors. • Main reason for discontinuation due to non-compliance with study protocol (3 consecutive days per month with <300 mL of milk consumed per day). • Majority of the AEs reported (93.1%) was not considered study related. • 5 AEs (4 for test and 1 for control) was considered

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
						likely related: lack of appetite for milk, regurgitation, dry skin, chronic diarrhea, abdominal pain. 1 AE was considered related (constipation). No further details were provided.
(Di Pierro *et al.*, 2012)	To determine the effect of *S. salivarius* K12 on reducing the incidence of streptococcal pharyngitis and/or tonsillitis and episodes of acute otitis media in children.	Open-label Study was not randomized and not blinded.	Children (M & F; 3 to 12 y) with and without recurrent streptococcal pharyngitis and/or tonsillitis $n_I = 82$ $n_F = 78$	**Test**: $5x10^9$ CFU/tablet **Control 1:** no intervention was administered to controls with recurrent illness **Control 2**: no intervention was administered to controls without recurrent illness One tablet was consumed daily ($5x10^9$ CFU/day).	90 days	• Test tablet was well tolerated and did not produce any side effects. • 4 subjects in the test group were excluded from the analyses because they did not adhere to the study protocol (*i.e.*, missed more than 20 days of treatment).
(Di Pierro *et al.*, 2013)	To determine the effect of *S. salivarius* K12 on reducing the incidence of streptococcal pharyngitis and/or tonsillitis in adults	Open-label Study was not randomized and not blinded.	Adults (M & F; 18 to 65 y) with recurrent oral streptococcal pharyngitis $n_I = n_F = 40$	**Test**: $5x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($5x10^9$ CFU/day).	90 days	• All 20 subjects receiving the test tablets completed the study (*i.e.*, no dropouts). • Test tablet was well tolerated with no treatment-related side effects reported.
(Di Pierro *et al.*, 2014)	To determine the effect of *S. salivarius* K12 on reducing the incidence of streptococcal and viral pharyngitis and/or tonsillitis in children.	Open-label Study was randomized but not blinded.	Children (M & F; 3 to 13 y) with recurrent oral streptococcal disorders $n_I = 61$ $n_F = 60$	**Test**: no less than $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($1x10^9$ CFU/day).	90 days	• The study authors reported that the test tablet was "*was well tolerated and without any side effects worth mentioning*". • 1 subject dropped out of the study immediately after enrolment due to the poor taste of the test product.

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
(Di Pierro, Di Pasquale *et al.*, 2015)	To conduct a pilot study investigating the effect of *S. salivarius* K12 in children with recurrent secretory otitis media	Open-label, single-arm	Children (M & F; 3 to 9 y) with recurrent secretory otitis media $n_i = n_f = 22$	**Test**: no less than $1x10^9$ CFU/tablet One tablet was consumed daily ($1x10^9$ CFU/day).	90 days	• *S. salivarius* K12 demonstrated a *"very good safety profile with no treatment-related side effects occurring and no subject drop out."* • Tolerability was rated as "good" and "very good" in 20 of the 22 subjects, and "acceptable" in the remaining 2 subjects.
(Di Pierro, Colombo *et al.*, 2016b)	To conduct a pilot study investigating the effect of *S. salivarius* K12 in preventing pharyngotonsillitis and other illnesses in children.	Open-label Study was randomized but not blinded.	Children (M & F; 3 to 10 y) with recurrent streptococcal pharyngotonsillitis $n_i = n_f = 124$	**Test**: no less than $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($1x10^9$ CFU/day).	90 days	• Study authors reported *S. salivarius* K12 had *"excellent tolerability and compliance"* and *"absence of side effects"*.
(Di Pierro, Colombo *et al.*, 2016a)	To determine whether *S. salivarius* K12 can reduce the incidence of streptococcal disease and acute otitis media in 3 y old children.	Open-label Study was randomized but not blinded.	Healthy children (M & F; 33 to 45 months) $n_i = n_f = 222$	**Test**: no less than $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($1x10^9$ CFU/day).	180 days	• All of the enrolled children completed the study. • Study authors reported: *"No apparent side effects were detected in the treated group either during treatment or follow-up* [3 months post-intervention]."
(Di Pierro *et al.*, 2018)	To determine whether *S. salivarius* K12 reduced the incidence of streptococcal and viral pharyngo-tonsillitis and acute otitis media in children.	Retrospective observational	Children (M & F; 3 to 14 y) with recurrent non-streptococcal infection n = 133	**Test**: no less than $1x10^9$ CFU/tablet One tablet was consumed daily ($1x10^9$ CFU/day).	90 consecutive days in 2 periods (Oct to Dec 2015; April to June 2016)	• Compliance and tolerability were reported to be "excellent". • Only 1 side effect was reported by the study authors. A 6-year old boy had a single episode of

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
						mild bronchospasm once after a few days of treatment with *S. salivarius* K12. It appears the subject continued with the study with no further incident.
(Doyle *et al.*, 2018)	To determine the effectiveness of *S. salivarius* K12 in preventing group A streptococcus pharyngitis in children.	Randomized, placebo-controlled, parallel Blinding NR.	Children at high risk of acute rheumatic fever (M & F; 5 to 14 y) n_i = 1314 n_f = 1137	**Test:** $2.5x10^9$ CFU/lozenge **Control**: matched placebo lozenge[b] The children received individual lozenges from school staff during the school day ($2.5x10^9$ CFU/day).	1 school year (max 209 days)	• Study authors did not report whether any AEs were observed by the participants. • In general, the lozenges were considered "well accepted", with only 2 children refusing to take them regularly.
(Gregori *et al.*, 2016)	To assess retrospectively whether *S. salivarius* K12 reduces the occurrence of pharyngo-tonsillar infections in children.	Retrospective observational	Children (M & F; 3 to 7 y) with recurrent group A beta-hemolytic streptococci pharyngo-tonsillar infections n = 130	**Test:** $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($1x10^9$ CFU/day).	90 days	• No child had to stop taking the test tablet before the study intervention period ended.
(Gilbey *et al.*, 2015) ***New study since GRN No. 807***	To investigate whether the supplementation of *S. salivarius* K12 to routine antibiotic therapy will affect the duration and symptom severity of acute pharyngotonsillitis.	Randomized, double-blind, placebo-controlled, parallel	Adults (M & F; 18 y and older) with severe acute pharyngotonsillitis n_i = 60 n_f = 53	**Test:** $2x10^9$ CFU/tablet **Control**: matched placebo tablet One tablet was taken twice daily ($4x10^9$ CFU/day).	10 days	• 7 participants (n=3 in test, n=4 in control) were excluded due to "noncompliance with the treatment". • Study authors did not report whether any AEs were observed by the participants.
(Jenks *et al.*, 2010)	To investigate whether supplementation of a multi-strain product (containing *S. salivarius*	Randomized, double-blind, placebo-	Adults (M & F; 18 y and older) with spondyloarthritis	**Test:** powder containing $1x10^8$ CFU/g of *S. salivarius* K12, $4x10^8$ CFU/g of *B. lactis* LAFTI B94, and $4x10^8$ CFU/g of	12 weeks	• All participants completed the study. • 14/32 (43.8%) in the test group and 12/31 (38.7%)

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
New study since GRN No. 807	K12) affects health outcomes in individuals with spondyloarthritis.	controlled, parallel	$n_i = n_f = 63$	*L. acidophilus* LAFTI L10 **Control**: matched placebo powder Participants were told to take 1 spoonful of powder (*ca.* 0.8 g) by mouth twice daily, corresponding to *ca.* 1.6×10^8 CFU/day of *S. salivarius* K12		in the placebo group reported AEs. All were rated as minor and self-limiting. • The incidence and types of AEs reported were similar between the test and control groups. Change in bowel habit was the most common AE in both groups (test: n=7; control: n=6). No serious AEs were observed. • NSD between groups in fecal calprotectin or change in bowel symptom questionnaire scores at end-of-study.
(He *et al.*, 2020) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* K12 on tongue-coating associated halitosis	Randomized, double-blind, placebo controlled, parallel	Adults (M & F; 23 to 44 y) with tongue-coating associated halitosis $n_i = 33$ $n_f = 28$	**Test:** 1×10^9 CFU/tablet **Control**: matched placebo tablet One tablet was taken twice daily (2×10^9 CFU/day).	30 days	• None of the participants experienced AEs • 5 participants were excluded from the study (n=3 in test; n=2 in control), with 1 (control) using antibiotics, and 4 being lost to follow-up.
(Horz *et al.*, 2007)	To determine the feasibility of using qPCR to assess the persistence of *S. salivarius* K12 in the oral cavity.	Not applicable (single subject)	Single healthy adult (M), 40 y old	Participant consumed lozenges containing 1×10^{10} CFU at 2h interval over 8h for 3 days. Total 4 lozenges consumed per day (4×10^{10} CFU/day).	3 days	• No AEs were reported by the participant during or after the trial.
(Hu *et al.*, 2019)	To evaluate the efficacy and safety of *S. salivarius* K12 as an adjuvant in treating oral	Randomized, double-blind, placebo	Adults (M & F; >18 y) with oral candidiasis $n_i = 56$	**Test**: $\geq 1 \times 10^9$ CFU/lozenge **Control**: matched placebo lozenge	4 weeks	• No severe AEs were reported. • 6 and 8 subjects in the test and control groups,

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
New study since GRN No. 807	candidiasis with nystatin.	controlled, parallel	n_f= 49 (safety-analyses)	Participants consumed 2 lozenges per day with nystatin tablets (2x10^9 CFU/day).		respectively, reported AEs. • Study authors noted: *"One patient complained borborygmus and pharyngeal discomfort in K12 group, and it was considered a possible drug-related adverse event."*
(Jamali *et al.*, 2016)	To evaluate the effect of *S. salivarius* K12 on oral malodor in children	Randomized, controlled, parallel	Children (M & F; 6 to 9 y) with an organoleptic score of 2 or more at baseline n_i = 208 n_f= 197	**Group A**: conventional oral hygiene practices (COH) **Group B**: COH + tongue scrapings (TS) **Group C**: chlorhexidine (CHX) + COH + TS **Group D**: CHX + COH + TS + *S. salivarius* K12 (>1x10^9 CFU/lozenge; 1 lozenge per day)	Unclear; appears to be as long as 3 months	• Study authors did not report whether any AEs were observed by the participants.
(Lee *et al.*, 2010)	Pilot study to investigate the effect of multi-strain blend on the metabolism of sulfasalazine.	Open-label, single-arm	Patients with rheumatoid arthritis taking stable doses of sulfasalazine (M & F; mean age = 56 y) $n_i = n_f$= 12	Participants consumed a powder blend (BioRestore®) containing *S. salivarius* K12 at 1x10^8 CFU, *L. acidophilus* L10 at 4x10^8 CFU, *B. lactis* B94 at 4x10^9 CFU. The powder was taken twice a day for total *S. salivarius* K12 of 2x10^8 CFU/day.	7 days	• 4 patients reported AEs at the end of the intervention period, including gastrointestinal disturbance (n=3) and a flareup of the rheumatoid arthritis (n=1). The AEs were reported as mild to moderate.
(Li *et al.*, 2020) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* K12 on symptomatic oral lichen planus	Randomized, non-blinded, controlled, parallel	Adults with oral lichen planus (M & F; 22 to 79 y) $n_i = n_f$= 40	**Test**: no less than 1x10^9 CFU/tablet **Comparator**: topical 0.1% triamcinolone acetonide dental paste *S. salivarius* K12 tablet was	4 weeks	• No adverse reactions were observed.

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
				taken twice daily ($2x10^9$ CFU/day).		
(Marini *et al.*, 2019) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* K12 in children with recurrent pharyngitis-tonsillitis.	Open-label Study was randomized but not blinded.	Children (M & F; 5 to 10 y) with recurrent pharyngitis-tonsillitis. $n_i = n_f = 100$	**Test:** BactoBlis® containing *S. salivarius* K12 (dose NR) **Control:** no intervention was administered to the control group	90 days	• Study authors did not report whether any AEs were observed by the participants.
(Passariello *et al.*, 2020) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* K12 on denture stomatitis	Open-label Study was randomized. Blinding NR.	Adults (M & F; 67 to 83 y) who are denture wearers. $n_i = n_f = 50$	**Test:** BactoBlis® containing *S. salivarius* K12 (10^9 CFU/tablet) **Control:** no intervention was administered to the control group 1 tablet was taken once daily (10^9 CFU/day)	30 days	• Study authors did not report whether any AEs were observed by the participants.
(Power *et al.*, 2008)	To investigate the extent of colonization of *S. salivarius* K12 in infants	Open-label, single-arm	Infants (age and sex NR) prone to otitis media scheduled to undergo ventilation tube placement. n = 19	**Test:** powdered formulation with *S. salivarius* K12 (reported as $1x10^{10}$ to $3.4x10^{10}$ CFU/day in GRN 581) 1 teaspoon was placed on the child's tongue twice daily.	10 days	• Study authors did not report whether any AEs were observed by the participants.
(Sarlin *et al.*, 2021) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* K12 on the nasopharyngeal and saliva microbiome in children.	Open-label Study was randomized. Microbiological analyses were blinded.	Children (M & F; 1 to 6 y) attending daycare centers. $n_i = 121$ n_f reported as number of biological samples collected at 1-month and 2-month time period	**Test (children ≤3y old):** powdered formulation with *S. salivarius* K12 ($5x10^9$ CFU/sachet) **Test (older children):** Chewable tablet containing *S. salivarius* K12 ($1x10^9$ CFU/tablet) **Control:** no intervention was administered to the control group	30 days	• Intervention with *S. salivarius* K12 did not alter the diversity of the nasopharyngeal or saliva microbiome. • Short-term increase in relative abundance of *S. salivarius* was observed in saliva of children receiving the *S. salivarius* K12 product.

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
				Daily dose provided was $5x10^9$ CFU/day (powder) and $1x10^9$ CFU/day (tablet).		
Studies Conducted with *S. salivarius* M18						
(Bardellini *et al.*, 2020) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* M18 on the reformation of black staining on the teeth of children.	Randomized, open label, controlled, parallel	Children (M & F; 4 to 10 y) with black teeth stains $n_i = 58$ (29/group) $n_f = 54$	**Test:** no less than $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group Test tablet was consumed once a day ($1x10^9$ CFU/day).	3 months	• 4 participants (n=1 in the test; n=3 in control) were excluded from the study because they started antibiotic therapy. • Study authors did not report whether any AEs were observed by the participants.
(Benic *et al.*, 2019) ***New study since GRN No. 807***	To investigate the effect of *S. salivarius* M18 on oral hygiene indices and halitosis in participants with orthodontic braces.	Randomized, triple-blind, placebo-controlled, parallel	Participants (M & F; 10 to 30 y) wearing orthodontic braces $n_i = n_f = 64$	**Test**: $3.6x10^9$ CFU/lozenge **Control:** matched placebo lozenge Two lozenges were consumed per day ($7.2x10^9$ CFU/day).	1 month	• Study authors reported that: *"No adverse events were recorded during the trial."*
(Burton, Wescombe *et al.*, 2013)	To evaluate the persistence of *S. salivarius* M18 in the oral cavity.	Randomized, parallel. Participants were blinded. Blinding of investigators was NR.	Healthy adults (18 y and older; average age = 19 y; gender NR) $n_i = 75$ n_f = NR	**Test 1**: $1x10^6$ CFU/lozenge **Test 2**: $1x10^7$ CFU/lozenge **Test 3**: $1x10^8$ CFU/lozenge **Test 4**: $1x10^9$ CFU/lozenge One lozenge was consumed daily ($1x10^9$ CFU/day).	28 days	• Study authors did not report whether any AEs were observed by the participants.
(Burton *et al.*, 2013)	To evaluate the effect of *S. salivarius* M18 in the prevention or reduction in the risk of dental caries in children.	Randomized, double-blind, placebo controlled, parallel	Children (M & F; 5 to 10 y) with a history of dental caries $n_i = 100$ $n_f = 83$	**Test**: $3.6x10^9$ CFU/lozenge **Control:** matched placebo lozenge Two lozenges were consumed per day ($7.2x10^9$ CFU/day).	3 months	• 11 participants dropped out from the study for the following reasons: did not like the taste of lozenges (n=6); protocol deviations (n=1); lost to follow-up (n=4). Data for 6 participants were excluded due to non-compliance (consumed <75% of the prescribed lozenges/month).

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
						• 4 cases of adverse reactions were reported (n=3 in test group; n=1 in control). The study authors indicated: *"None of the adverse events resulted in the participants leaving the trial, and none was of a serious nature."* No further details were provided in the publication[b].
(Campanella *et al.*, 2018) ***New study since GRN No. 807***	To evaluate the effect of an multi-strain product affects the incidence of acute oral and respiratory tract infections in a pediatric population.	Randomized, double-blind, placebo controlled, parallel	Children (M & F; 12 to 15 y) with recent clinical history of oral and respiratory tract infections $n_I = n_P = 40$	**Test**: PRO-Kids ENT Hyperbiotics containing *S. salivarius* K12, *S. salivarius* M18, *L. reuteri*, *L. sakei*, and *L.* paracasei **Control**: matched placebo Participants consumed 3 tablets per day during the first month of the study, followed by 1 tablet per day for the remaining 2 months. Dose NR.	3 months	• Study authors did not report whether any AEs were observed by the participants.
(Di Pierro, Zanvit *et al.*, 2015)	To evaluate the safety and tolerability of *S. salivarius* M18 and its effects on caries formation in children.	Open-label Study was randomized but not blinded.	Children (M & F; 6 to 17 y) at high risk for dental caries $n_I = n_P = 76$	**Test:** no less than $1x10^9$ CFU/tablet **Control:** no intervention was administered to the control group One tablet was consumed daily ($1x10^9$ CFU/day).	90 days	• No dropouts occurred in this study. • *S. salivarius* M18 demonstrated a *"very good safety profile with no treatment-related side effects and no subject dropout"*. • Tolerability was assessed as "good" and "very good" in 35 of the 38 subjects, and as "acceptable" in 3 subjects.

Reference	Study Objective	Study Design	Study Population	Test Articles and Administration Levels	Duration of Intervention	Safety-Related Outcomes
(Vesty *et al.*, 2020) ***New study since GRN No. 807***	To evaluate the effect of *S. salivarius* M18 in head and neck cancer patients post-radiotherapy	Randomized, double-blind, placebo-controlled	Adults (M & F; mean age 53.5 y in placebo, 53.3 y in test) who had received radiotherapy in the previous 6 months n_I = 17 n_f = 13	Test: $3.5x10^9$ CFU/lozenge Control: matched placebo lozenge One lozenge was consumed daily ($3.5x10^9$ CFU/day).	4 weeks	• 3 subjects in the placebo group and 1 subject in the test group withdrew from the study. 2 subjects were lost to follow-up, 1 had received antibiotic treatment, and 1 had failure to comply (did not consume lozenges). • Study authors did not report whether any AEs were observed by the participants.

AEs = adverse events; CFU = colony forming units; CHX = chlorhexidine; F = females; GI = gastrointestinal; GSRS = Gastrointestinal Symptom Rating Scale; M = males; n_f = number of participants completing the study; n_I = number of participants randomized into the study; NR = not reported; NSD = no statistically significant difference; SS = statistically significant; VAS = visual analogue scale; y = years.

[a] The placebo lozenge was reported to contain trace amount of *S. salivarius* K12 (<$2.5x10^4$ CFU/lozenge). The study authors noted: *"Children in the placebo group in this study received a small dose of S. salivarius K12 due to contamination of the lozenge production facility, information that we were only made aware of after the trial had commenced."*

[b] In the GRAS notice submitted by BLIS Technologies for *S. salivarius* M18 (GRN No. 807), it was further elaborated that: *"Four cases of adverse reactions were reported, specifically, 3 events in the S. salivarius M18 group included a sore throat and 2 cases of chickenpox, while 1 bleeding gum event occurred in the placebo group. None of the adverse events were considered serious or related to the treatment. No subject left the trial as a result."*

6.4.3 Case Reports of Human Infections Associated with *S. salivarius*

It is recognized that most microorganisms are harmless for healthy individuals; however, in some instances, these microbes (including commensal bacteria) can produce opportunistic infections (Pariza *et al.*, 2015). This can occur when tissue sites that are normally protected by host barriers (*e.g.*, skin, mucous membranes) are broken (*e.g.*, from a wound), or in those with weakened immune systems (Pariza *et al.*, 2015).

S. salivarius is a commensal organism that occurs prominently in the oral cavity and gastrointestinal tract. Although *S. salivarius* frequently enter the bloodstream, infections with *S. salivarius* are considered rare due to their low virulence (Public Health Agency of Canada, 2018). Nonetheless, case reports of infections associated with *S. salivarius* have been published in the literature, as discussed extensively in the GRAS notices for *S. salivarius* K12 and M18 (GRN No. 591 and 807) and incorporated by reference herein. These case reports are almost exclusively iatrogenic in nature, being typically related to infection following surgical intervention with poor hygiene control, or they were reported to occur following major tissue trauma or in immunocompromised individuals (GRN No. 591 and 807). As stated in the GRAS Panel Statement for *S. salivarius* K12 (GRN No. 591), which was reiterated for *S. salivarius* M18 (GRN No. 807):

> *"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)."*

Additionally, as concluded in the GRAS notices for *S. salivarius* K12 and M18 (GRN No. 591 and 807), there do not appear to be clusters of *S. salivarius* strains with pathogenic or unique opportunistic phenotypes that exist for the species. Genomic analyses of various clinical isolates and commensal *S. salivarius* from healthy individuals have revealed no clear clustering of the strains in the phylogenetic tree, suggesting that the infection-associated strains were opportunistic rather than pathogenic in nature (Chaffanel *et al.*, 2015; Delorme *et al.*, 2007; Delorme *et al.*, 2015).

An updated search of the literature identified several additional case reports describing *S. salivarius* isolates in clinical infections among compromised individuals or from iatrogenic causes[3] (Ansari *et al.*, 2018; Barajas-Colon & Warady, 2021; Domínguez-Domínguez *et al.*, 2017; Hevroni *et al.*, 2020; Jovanovic *et al.*, 2019; Jun, 2019; Lechner *et al.*, 2020; Mehanna *et al.*, 2021; Oblitas *et al.*, 2020; Olson *et al.*, 2019; Vargas Osorio *et al.*, 2019). Overall, the available data continue to support the conclusions derived in GRN No. 591 and 807 that *S. salivarius*, similar to other microbial cultures commonly used in the food supply (*e.g.*, lactobacilli, bifidobacteria), are generally innocuous in nature but may result in opportunistic infections under rare circumstances. Moreover, *in silico* analyses have demonstrated that the genome of *S. salivarius* DB-B5 does not contain any of the virulence factors that have been described for pathogenic streptococci (see Section 6.5.2).

[3] Only case reports that are published in English are included here.

6.5 *IN SILICO* ANALYSES

6.5.1 Genomic Analyses for Antibiotic Resistance Genes

The genome sequence of *S. salivarius* DB-B5 was screened for genes involved in antibiotic resistance using the Comprehensive Antibiotic Resistance Database (CARD)[4] (Jia *et al.*, 2017). CARD is an online bioinformatic database of antibiotic resistance determinants organized through the Antibiotic Resistance Ontology. To search for the presence of potential antibiotic resistance genes, the protein sequences of all predicted open reading frames (ORF) of *S. salivarius* DB-B5 was entered into the Resistance Gene Identifier (RGI) tool on the CARD website. The generated output of hits is defined as: "Perfect", meaning the sequences are 100% identical to the CARD reference sequence; "Strict", meaning the match bitscore are above the curated BLAST bitscore cutoff; and "Loose", meaning the match bitscore are below the curated BLASTP bitscore cutoff.

None of the ORFs from *S. salivarius* DB-B5 had "Perfect" or "Strict" hits against the antibiotic resistance sequences in the CARD database. RGI predicted 177 ORFs as "Loose" hits, which are sequences outside the detection model cut-offs, and generally indicates distant homologs or spurious partial hits that may not have a role in antibiotic resistance (Jia *et al.*, 2017). Analysis of the "Loose" hits shows that the percent identities towards the resistance genes of the Antibiotic Resistance Ontology are extremely low (most are in the 20% to 40% range) and/or the bit score are low and far removed from the bit score cut-offs, indicating that the hits are unlikely to be true hits of significance. An Excel spreadsheet containing the full details of these "Loose" hits is publicly available (Li *et al.*, 2021)[5].

As an additional measure, the protein sequences were run on BlastKOALA[6] to determine if any of the predicted ORFs were involved in antimicrobial resistance pathways (Kanehisa *et al.*, 2016; Kanehisa, 2018). BlastKOALA is an annotation server that assigns KO (KEGG [Kyoto Encyclopedia of Genes and Genomes] Orthology) to genes which allows for reconstruction of KEGG pathways and BRITE hierarchies to infer high-level functions of the input organism (Aoki-Kinoshita & Kanehisa, 2007). In this analysis, 59.6% of the amino acid sequences, corresponding to 1216 proteins, were annotated with KOs. BRITE mapping to ko01504 (antimicrobial resistance genes) identified 3 KO annotations of interest: K05593, K17836, and K07260. K05593 corresponds to *aadK*, a nucleotidyltransferase which may be involved in aminoglycoside resistance. The gene annotated as K05593 (locus tag HRE60_02705) was also identified in CARD as *aadK*, however, its low percent identity (34.53%) and bit score (171.8, against a pass big score of 500), indicates that this gene is likely not a true hit to *aadK*, as confirmed through the susceptibility of *S. salivarius* DB-B5 to the aminoglycosides, kanamycin, gentamicin, and streptomycin in phenotypic assays (see Section 6.6). K17836 corresponds to *penP*, whose gene product is involved in beta-lactam resistance, as well as penicillin and cephalosporin biosynthesis. Only one of the 4 KOs involved in the class A beta-lactam resistance was present. K07260 corresponds to zinc D-Ala-D-Ala carboxypeptidase, a protein involved in the normal peptidoglycan biosynthetic pathway. The enzyme is considered an accessory gene in vancomycin resistance pathways, and not involved in the actual

[4] https://card.mcmaster.ca/
[5] See Supplementary Table 1 of this publication.
[6] https://www.kegg.jp/blastkoala/

resistance to the antibiotic. No other genes were detected within the vancomycin resistance pathway, indicating that *S. salivarius* DB-B5 does not carry any resistance mechanism for vancomycin.

These *in silico* analyses demonstrates the absence of functional and transferrable antibiotic resistance genes in *S. salivarius* DB-B5, which is further confirmed by phenotypic testing demonstrating the strain is sensitive to a diverse range of antibiotic classes, as described further below in Section 6.6.

6.5.2 Genomic Analyses for Virulence Factors

The Virulence Factor Database (VFDB) was used to screen for potential virulence factors in the *S. salivarius* DB-B5 genome (Li *et al.*, 2021). The VFDB core database was downloaded (Chen *et al.*, 2015), and a local reciprocal BLASTP analysis was performed against the protein sequences of all predicted ORFs of *S. salivarius* DB-B5 using the BLAST+ software (Camacho *et al.*, 2009). Greater than 50% identity match and E-values of less than 10^{-5} were used as cut-off values. A total of 15 hits were identified (see Table 6.5.2-1).

The VFDB hit genes were assessed for their virulence potential. A reciprocal BLASTP against the nr database on NCBI was performed on the hit genes to determine their predicted role and function. A reciprocal blast was also performed on the publicly available genomes of 7 commercial live microbial strains to determine the presence of any homologues of the VFDB hit genes. The 7 selected strains included: *B. longum* 35624, *L. helveticus* R0052, *L. reuteri* SD2112/ATCC 55730, *L. rhamnosus* GG, *L. rhamnosus* R0011, *S. salivarius* K12, and *S. salivarius* M18.

As indicated in Table 6.5.2-1, the 15 identified genes are commonly found in many bacteria and encode for proteins involved in normal metabolic processes. Some of the matches identified in the VFDB are part of gene clusters involved in the biosynthesis of virulence factors only in specific taxa. For instance, an ORF in *S. salivarius* DB-B5 had a positive hit against a gene identified as UDP-glucose pyrophosphorylase by VFDB is involved in hyaluronic acid capsule biosynthesis in *S. pyogenes* (Group A Streptococcus). However, the same gene has a better hit with UTP-glucose-1-phosphate uridylyltransferase according to the nr database on NCBI BLASTP, which is a protein required for glycogenesis and cell wall metabolism in most bacteria. Furthermore, all other genes involved in hyaluronic acid capsule biosynthesis are absent in the *S. salivarius* DB-B5 genome.

Lastly, the 15 genes with positive hits to the VFDB were also present in the commercial strains, including those deemed safe for human consumption. Thus, the identified genes are not considered to pose any safety concerns with respect to virulence potential.

Table 6.5.2-1 Analysis for Genes in *S. salivarius* DB-B5 with Hits to VFDB

DB-B5 locus tag	BLAST hit (nr)	BLAST E-value (%ID)	VFDB gene hit	VFDB E-value (%ID)	Found in Commercial Strains[a]	Analysis of BLAST hit
HRE60_08810	UTP-glucose-1-phosphate uridylyltransferase [*S. salivarius*]	0 (100%)	UDP-glucose pyrophosphorylase [Hyaluronic acid capsule - *S. pyogenes*]	0 (88.7%)	yes (7/7)	Hyaluronic acid capsule is a virulence factor in Group A Strep only. This gene is found in most bacteria for glycogenesis and cell wall metabolism. Other genes of hyaluronic acid capsule biosynthesis are not present.
HRE60_06080	peptide-methionine (R)-S-oxide reductase [*S. salivarius*]	0 (97.2%)	trifunctional thioredoxin/methionine sulfoxide reductase [*N. meningitidis*]	3.89E-131 (55.8%)	yes (7/7)	Normal stress-related protein found in most bacteria.
HRE60_05360	N-acetylmuramidase [*Streptococcus* sp.]	8.78E-170 (99.2%)	autolysin [*L. monocytogenes*]	2.26E-40 (51.0%)	yes (6/7)	Normal hydrolase of peptidoglycan. Conserved domain is different from autolysin of *L. monocytogenes*.
HRE60_05035	DUF814 domain-containing protein [*S. salivarius*]	0 (99.5%)	fibronectin-binding protein [*S. pyogenes*]	0 (76.9%)	yes (6/7)	Normal component of the ribosome quality control complex that binds fibronectin/fibrinogen.
HRE60_04280	UDP-glucose 4-epimerase GalE [*Streptococcus* sp.]	0 (100%)	UDP-glucose 4-epimerase [LOS - *H. influenzae*]	1.19E-153 (60.6%)	yes (7/7)	Found in all bacteria - epimerase for galactose and glucose. Also used in LPS/LOS biosynthesis, n/a in *Streptococcus* spp.
HRE60_03525	metal ABC transporter substrate-binding [*S. salivarius*]	0 (99.7%)	Mn-binding adhesion; Mn ABC transporter [*S. pneumoniae*]	0 (81.5%)	yes (7/7)	Found in most bacteria. The protein may act as an adhesin.
HRE60_01785	3-hydroxyacyl-ACP dehydratase [*Streptococcus* sp.]	7.03E-98 (100%)	(3R)-hydroxymyristoyl ACP dehydratase [LPS - *B. melitensis*]	1.36E-42 (50.8%)	yes (6/7)	Part of normal fatty acid biosynthesis in bacteria. Not related to virulence in *Streptococcus*.
HRE60_02780	Clp protease ATP-binding subunit [*S. salivarius*]	0 (99.4%)	Clp protease [*L. monocytogenes*]	0 (60.0%)	yes (7/7)	Heat shock protein found in most bacteria. Clp protease is important in *L. monocytogenes'* intracellular survival. No sign of contribution to virulence in non-intracellular species.
HRE60_01580	Clp protease proteolytic subunit [*Streptococcus* sp.]	1.14E-142 (100%)	Clp protease proteolytic subunit [*L. monocytogenes*]	3.16E-91 (63.5%)	yes (7/7)	
HRE60_04615	UDP-galactopyranose mutase [*S. salivarius*]	0 (100%)	UDP-galactopyranose mutase [*E. faecalis*]	3.95E-170 (62.8%)	yes (4/7)	Genes found in most bacteria for phospholipid metabolism.
HRE60_00955	phosphatidate cytidylyltransferase [*Streptococcus* sp.]	0 (99.6%)	phosphatidate cytidylyltransferase [*E. faecalis*]	1.03E-88 (51.7%)	yes (7/7)	

DB-B5 locus tag	BLAST hit (nr)	BLAST E-value (%ID)	VFDB gene hit	VFDB E-value (%ID)	Found in Commercial Strains[a]	Analysis of BLAST hit
HRE60_04575	sugar transferase [*S. salivarius*]	0 (99.8%)	glycosyl transferase CpsE [Capsule - *S. agalactiae*]	1.66E-163 (50.4%)	yes (7/7)	Cps genes in *S. agalactiae* is involved in the biosynthesis of type III capsular polysaccharide, considered virulent only in Group B Strep.
HRE60_04570	tyrosine protein kinase [*S. salivarius*]	0 (100%)	CpsD autokinase [Capsule - *S. agalactiae*]	1.83E-99 (61.3%)	yes (7/7)	
HRE60_04560	tyrosine protein phosphatase [*S. salivarius*]	0 (99.6%)	CpsB phosphatase [Capsule - *S. agalactiae*]	1.48E-130 (71.6%)	yes (6/7)	
HRE60_00985	chaperonin GroEL [*Streptococcus* sp.]	0 (100%)	Hsp60 heat shock protein [*L. pneumophila*]	0 (58.1%)	yes (7/7)	Found in most bacteria. Not related to virulence in *Streptococcus*.

ORF = open reading frame; VFDB = Virulence Factor Database.

[a] The following commercialized live microbial strains were screened: *B. longum* 35624, *L. helveticus* R0052, *L. reuteri* SD2112/ATCC 55730, *L. rhamnosus* GG, *L. rhamnosus* R0011, *S. salivarius* K12, and *S. salivarius* M18.

6.5.3 Detection of Mobile Genetic Elements

Although no genes of concerns were identified with respect to antimicrobial resistance and virulence factors, the genome of *S. salivarius* DB-B5 was searched for the presence of mobile genetic elements (MGEs) as an added precaution. The whole genome nucleotide sequence was inputted into MobileElementFinder[7], a web-based tool that identifies MGEs and their relation to antimicrobial resistance genes and virulence factors (Johansson *et al.*, 2020). Two Insertion Sequences (IS) were detected on the chromosome; these were identified as IS*Str1*, which is a member of the IS200 family, and is detected in other *S. salivarius* strains (Fléchard *et al.*, 2019). Importantly, the search did not identify any putative antibiotic resistance genes or virulence genes near the IS. The search also confirmed the absence of other types of MGEs, including conjugative MGEs.

6.6 Antibiotics Susceptibility Test

S. salivarius DB-B5 was assessed for its susceptibility to a range of antibiotics using Etest® strips by bioMérieux Inc., according to the instructions provided. The minimum inhibitory concentration (MIC) was measured by identifying the zone of inhibition that intersects the strip. As confirmatory analysis, the MICs were also determined in accordance with the broth microdilution method in ISO 10932:2010 (IDF 223:2010). As shown in Table 6.6-1, *S. salivarius* DB-B5 was found to be susceptible to all antibiotics tested, according to both the European Food Safety Authority (EFSA)'s breakpoints for *S. thermophilus* (EFSA FEEDAP, 2018), and the Clinical and Laboratory Standards Institute (CLSI)'s breakpoints for viridans streptococci (CLSI, 2020).

Table 6.6-1 Results of Antibiotic Resistance Test Conducted for *S. salivarius* DB-B5

Antibiotic	*S. salivarius* DB-B5 MIC from Etest® (µg/mL)	MIC from Broth Microdilution (µg/mL)	EFSA's Breakpoint for *S. thermophilus* (µg/mL)	CLSI's Breakpoint for viridans streptococci (µg/mL)
Ampicillin	0.125	0.25	2	8
Ceftriaxone	Not tested	<0.0625	Not listed	4
Chloramphenicol	2	2	4	16
Clindamycin	0.064	1	2	1
Erythromycin	0.064	0.03125	2	1
Gentamicin	6	8	32	Not listed
Kanamycin	Not tested	64[a]	Not required	Not listed
Vancomycin	1	1	4	Not listed
Penicillin	0.19	0.0625	Not listed	4
Streptomycin	32	16	64	Not listed
Tetracycline	0.25	0.25	4	8

MIC = minimum inhibitory concentration.

[a] No breakpoints were established for kanamycin by EFSA or the CLSI. However, the MIC for *S. salivarius* DB-B5 is identical to the MIC for kanamycin reported in GRN No. 807 for *S. salivarius* M18 (64 µg/mL).

[7] https://cge.cbs.dtu.dk/services/MobileElementFinder/

6.7 Additional Considerations

6.7.1 Production of Antimicrobials

In addition to enhancing the sensory qualities of foods, fermentation has long been used as a method of food preservation by preventing the growth of food-borne pathogens (Şanlier *et al.*, 2019; Tamang *et al.*, 2016). It is well known that many lactic acid bacteria, including those isolated from fermented vegetables and milk products, produce substances that can inhibit the growth of other microorganisms (Moradi *et al.*, 2020; Silva *et al.*, 2020; Tamang *et al.*, 2016). Of these substances, there is particular interest in the antimicrobial properties of bacteriocins, which are a diverse group of ribosomally synthesized peptides (Chikindas *et al.*, 2018; Yang *et al.*, 2014). Different classification systems have been proposed for bacteriocins over the years, based according to their size, structures, and modes of action (Chikindas *et al.*, 2018; Heng & Tagg, 2006; Soltani *et al.*, 2020). The bacteriocins produced by the Gram-positive lactic acid bacteria, particularly the lantibiotics (Class I bacteriocins), are amongst those that have been the most well-studied (Barbour *et al.*, 2020; López-Cuellar *et al.*, 2016). Lantibiotics are polycyclic peptides characterized by the presence of lanthionine and/or β-methyllanthionine, which are unusual amino acids formed through post-translational modifications (Barbour *et al.*, 2020; Wescombe *et al.*, 2009).

Bacteriocins have been developed for use in the food supply. For instance, the lantibiotic nisin (INS No. 234), which comprise a mixture of antimicrobial polypeptides (34 amino acids in length), is widely accepted for use as a food additive, specifically as an antimicrobial preservative in foods[8]. Nisin is produced by certain strains of *Lactococcus lactis* subsp. *lactis* (EFSA, 2006). Moreover, other bacteriocins, including recombinant colicins and salmocins, have been concluded GRAS for use as antimicrobial preservatives in foods (*e.g.*, GRN Nos. 593, 676, 775, 824). The commercially available *S. salivarius* K12 and M18 are also known to produce bacteriocins. Both strains have been shown to inhibit oral pathogens such as *Porphyromonas gingivalis*, *Porphyromonas canoris*, and *Prevotella intermedia*, and each strain has their own extended spectrum of antibacterial activity towards other organisms (Barbour *et al.*, 2020; Wescombe *et al.*, 2012). Production of bacteriocins is known to be widespread among the *S. salivarius* species. For instance, the gene encoding the lantibiotic salivaricin A (*salA*) has been detected in 11 out of the 18 *S. salivarius* strains tested by PCR analysis (Dierksen *et al.*, 2007). Additionally, production of the streptococcal lantibiotics salivaricin A variants, salivaricin B, streptin, and/or SA-FF22 were detected in 9 of 28 *S. salivarius* strains tested (Wescombe *et al.*, 2006). It has been reported that *S. salivarius* K12 produces 2 lantibiotics (salivaricins A2 and B) (Hyink *et al.*, 2007), whereas *S. salivarius* M18 produces 4 lantibiotics (salivaricins A2, 9, MPS, and M) (Heng *et al.*, 2011).

The genome of *S. salivarius* DB-B5 was found to contain 2 separate bacteriocin biosynthetic clusters, including a thiazolyl peptide bacteriocin locus on the megaplasmid and a *blpU* bacteriocin locus on the chromosome (Fields *et al.*, 2020). Based on its sequence, the thiazolyl peptide bacteriocin locus likely encodes a putative lantibiotic. With respect to the *blpU* loci, in addition to the production of lantibiotics, many *Streptococcus* species (including *S. salivarius* and *S. thermophilus*) have bacteriocin-encoding genes that are under the control of the *blp* (bacteriocin-like peptides) system, which is

[8] For example, nisin is approved in Canada (*List of Permitted Preservatives*), U.S. (21 CFR §184.1538; GRN No. 65), and the European Union (Regulation (EC) No 1333/2008). Nisin is also included in the Codex Alimentarius General Standard for Food Additives (CODEX STAN 192-1995).

regulated through a quorum-sensing mechanism (Hols *et al.*, 2019; Mignolet *et al.*, 2018; Wang & Dawid, 2018).

Overall, many commensal streptococcal species naturally reside within a competitive, polymicrobial niche, and bacteriocin production has evolved as a defense mechanism to inhibit competing organisms (Hols *et al.*, 2019; Wang & Dawid, 2018). Thus, bacteriocin production is known to be widespread amongst the commensal *S. salivarius* strains in the oral cavity (Wescombe *et al.*, 2009; Wescombe *et al.*, 2012). It is also important to recognize that many lactic acid bacteria, including those with a long history of use in food fermentation (*e.g.*, *S. thermophilus*), produce a diverse range of bacteriocins (EFSA, 2006; Kaškonienė *et al.*, 2017; Uriot *et al.*, 2017), and these have been consumed widely without cause for concern. Therefore, although *S. salivarius* DB-B5 may have the potential to produce antimicrobial compounds, this characteristic is not considered a novel trait, nor does it pose a safety risk.

6.7.2 Production of Biogenic Amines

Consumption of foods with high concentrations of biogenic amines may result in undesirable symptoms such as headaches, nausea or vomiting, alterations in blood pressures, and rashes (Barbieri *et al.*, 2019; EFSA BIOHAZ Panel, 2011; Pradhan *et al.*, 2020). Biogenic amines are low molecular weight nitrogenous compounds formed by the decarboxylation of amino acids by microbial species, including certain lactic acid bacteria (Barbieri *et al.*, 2019; Özogul & Özogul, 2019). As examples, the biogenic amines histamine, tyramine, putrescine, and cadaverine are formed from the decarboxylation of histidine, tyrosine, ornithine, and lysine, respectively (Barbieri *et al.*, 2019). In addition to decarboxylase enzymes, a system of active transporters (such as antiporter proteins) is required to allow for the uptake of the amino acid substrate into the cell, and to excrete the biogenic amine product (Barbieri *et al.*, 2019; EFSA BIOHAZ Panel, 2011). Various lactic acid bacteria present in fermented foods (*e.g.*, lactobacilli and *S. thermophilus*) are reported to harbor the genes encoding for these decarboxylases, and to exhibit the capacity for biogenic amine production (Barbieri *et al.*, 2019; Pradhan *et al.*, 2020).

Dose Biosystems has employed an *in silico* approach to identify possible genetic determinants for the synthesis of biogenic amines within the genome of *S. salivarius* DB-B5. The amino acid sequences of all predicted ORFs were run on BlastKOALA (Kanehisa *et al.*, 2016), and the pathways involved in biogenic amine production were analyzed. *S. salivarius* DB-B5 did not encode for any of the decarboxylase enzymes examined, which included: histidine decarboxylase, tyrosine decarboxylase, lysine decarboxylase, (hydroxy)tryptophan decarboxylase, ornithine decarboxylase, spermidine synthase, carboxynorspermidine synthase + decarboxylase, and spermine synthase (Li *et al.*, 2021). These enzymes contribute to the production of histamine, tyramine, cadaverine, tryptamine, serotonin, putrescine, spermidine, and spermine. Additionally, an *in vitro* assay was performed where *S. salivarius* DB-B5 was streaked onto decarboxylase media plates containing LB broth (pH 5.0) supplemented with 0.25% glycerol, 0.1% precursor amino acid (*i.e.*, histidine, tyrosine, lysine, tryptophan, 5-hydroxytryptophan, or ornithine/arginine), and 0.006% bromcresol purple (*i.e.*, a pH color indicator). Production of biogenic amines, which is phenotypically detected by a change in the plate coloration, was not observed for *S. salivarius* DB-B5 (Li *et al.*, 2021).

6.8 PARIZA DECISION TREE

Pariza and colleagues have developed a decision tree consisting of 13 questions to assess the safety of microbial cultures intended for human (and animal) consumption (Pariza *et al.*, 2015). Using this decision tree approach, *S. salivarius* DB-B5 can be concluded safe for consumption as a food ingredient (see Table 6.8-1).

Table 6.8-1 Pariza Decision Tree for Determining the Safety of Microbial Cultures Applied to *S. salivarius* DB-B5 (Pariza *et al.*, 2015)

#	Decision Tree Question[a]	Response
1.	Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).	**YES.** The taxonomic identity of *S. salivarius* DB-B5 has been confirmed by genomic analysis. The functional characteristics of the strain are also similar to other *S. salivarius* strains.
2.	Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)	**YES.** The genome of *S. salivarius* DB-B5 has been fully sequenced and is publicly available.
3.	Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)	**YES.** Bioinformatic analyses of the *S. salivarius* DB-B5 genome demonstrate that it does not contain classical *Streptococcus* virulence factors and/or toxins associated with pathogenicity.
4.	Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)	**YES.** Bioinformatic analyses of the *S. salivarius* DB-B5 genome, together with phenotypic testing for antibiotic susceptibility, demonstrate the absence of transferable antibiotic resistance genes in the strain.
5.	Does the strain produce antimicrobial substances? *Note: In this context, the term 'antimicrobial substances' refers to antibiotics that are used in medical or veterinary medicine.* (If NO, go to 6. If YES, go to 15.)	**NO.** Similar to other commensal *S. salivarius* strains, and various lactic acid bacteria present in fermented foods, *S. salivarius* DB-B5 does have the potential to produce bacteriocins. However, in the context of this question, these bacteriocins are not antibiotics that are used in medical or veterinary medicine.
6.	Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a. If NO, go to 8a)	**NO.**
7a.	Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food? (If YES, go to 8a. If NO, the expressed product(s) must be shown to be safe before proceeding to 8a.)	*Not applicable.*
8a.	Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? (If YES, go to 9a. If NO, go to 13a.)	**NO.** *S. salivarius* DB-B5 is a human commensal that was isolated from the supragingival plaque of a healthy female adult donor. Moreover, *S. salivarius* and the closely related *S. thermophilus* (previously *S. salivarius* subsp. *thermophilus*) have a history of safe use in food production. Thus, it is considered appropriate to proceed to question 9a.
9a.	Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been	**YES.** *S. salivarius* is included in the IDF/EFFCA's *Inventory of microbial food cultures with safety demonstration in fermented food products.* Moreover, the closely related *S. thermophilus*

#	Decision Tree Question[a]	Response
	affirmed to be safe for food use by an authoritative group of qualified scientific experts? (If YES, go to 10a. If NO, go to 13a.)	(previously *S. salivarius* subsp. *thermophilus*) is included in EFSA's list of microorganisms with QPS status. Other commercial strains, *S. salivarius* K12 and M18, also have GRAS status for uses in foods in the U.S. (see GRN No. 591 and 807).
10a.	Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food? (If YES, go to 11a. If NO, go to 13a.)	**YES.**
11a.	Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12a. If YES, go to 13a.)	**NO.** *S. salivarius* DB-B5 is intended for use as a general food ingredient, including addition to foods that are beyond the "traditional" fermented foods in which *S. salivarius* and *S. thermophilus* are typically found. Nevertheless, ingestion of *S. salivarius* strains is ubiquitous from the swallowing of saliva, which contains *S. salivarius* at approximately 10^7 to 10^8 CFU/mL. Transfer of *S. salivarius* strains between humans also occur regularly through normal social interactions.
12a.	Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14a. If YES, go to 13a.)	**NO.** The intended food uses of *S. salivarius* DB-B5 are comparable to those described for *S. salivarius* K12 and M18 strains in GRN No. 591 and 807. The estimated daily intake of *S. salivarius* DB-B5 from its intended uses in foods is expected to be within the ranges of those resulting from the use of the *S. salivarius* K12 and M18 strains, and to other commercialized live microbial strains in the food supply.
13a.	For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? If yes, go to 15. If no, go to 14a.)	**NO.** *S. salivarius* DB-B5 has been safely consumed by humans without adverse effects in 2 randomized, double-blinded, placebo-controlled clinical trials.
14a.	The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.	**Based on the decision tree, *S. salivarius* DB-B5 is concluded safe for its intended use in foods.**

[a] Adapted from Table 1 of Pariza *et al.* (2015). The Decision Tree also includes questions related to the use of the microbial cultures in animal feeds, which are not presented here.

6.9 SUMMARY

The information presented herein demonstrates that *S. salivarius* DB-B5 is safe for its intended conditions of use as a general food ingredient in conventional foods. All pivotal data pertinent to the safety evaluation of *S. salivarius* DB-B5 is in the public domain. Overall, the safety of *S. salivarius* DB-B5 is supported on the following basis:

- *S. salivarius* is a predominant member of the commensal oral microbiota in humans. The *S. salivarius* DB-B5 strain was isolated from the supragingival plaque of a healthy female adult donor, and it is not genetically modified.

- *S. salivarius* and the closely related *S. thermophilus* (previously *S. salivarius* subsp. *thermophilus*) have a history of safe consumption from fermented foods. *S. salivarius* is included in the IDF/EFFCA's *Inventory of microbial food cultures with safety demonstration in fermented food products*. Moreover, *S. thermophilus* is included in EFSA's list of microorganisms with QPS status. *S. salivarius* DB-B5 is manufactured in accordance with GMP and HACCP, using materials and processes that are commonly employed by the industry.

- The strain has been well characterized. Its genome has been fully sequenced, and genomic analysis has confirmed the taxonomic placement of the strain as a *S. salivarius* species.

- The functional characteristics of *S. salivarius* DB-B5 are similar to other *S. salivarius* strains. No unusual metabolic capabilities were observed for *S. salivarius* DB-B5 when its carbohydrate fermentation and enzymatic activity profiles were assessed using the API 50CH test strips and the API 20 Strep test kit. Although *S. salivarius* DB-B5 displayed weak alpha hemolysis, this same phenotype was observed for the commercially available *S. salivarius* K12 and M18 strains when tested under the same conditions.

- Bioinformatic analysis of the *S. salivarius* DB-B5 genome demonstrates the absence of transmissible antibiotic resistance genes or virulence factors. Phenotypic testing further showed *S. salivarius* DB-B5 to be susceptible to clinically relevant antibiotics.

- Consumption of *S. salivarius* DB-B5 was safe and well tolerated in 2 randomized, double-blind, placebo-controlled clinical studies.

- *S. salivarius* DB-B5 is intended for addition to comparable food categories and inclusion levels as other commercialized strains from this species with GRAS status (*S. salivarius* K12 and M18), and the intended uses of *S. salivarius* DB-B5 as a general food ingredient is not expected to materially increase the intake of live microbial cultures from the diet.

6.10 Conclusions

The data and information described herein demonstrate that *S. salivarius* DB-B5, meeting appropriate food-grade specifications and manufactured in accordance with cGMP, is safe for its intended conditions of use as a general food ingredient in conventional foods in the U.S (excluding infant formula and meat and poultry products regulated by the FSIS of the USDA), at levels providing a minimum $1x10^9$ CFU/serving. The data and information also demonstrate the intended uses for *S. salivarius* DB-B5, as described herein, is GRAS based on scientific procedures.

7. REFERENCES (21 CFR §170.255)

Abdelgadir, W. S., Hamad, S. H., Møller, P. L., & Jakobsen, M. (2001). Characterisation of the dominant microbiota of Sudanese fermented milk Rob. *International Dairy Journal, 11*(1), 63-70. 10.1016/S0958-6946(01)00042-5

Abranches, J., Zeng, L., Kajfasz, J. K., Palmer, S. R., Chakraborty, B., Wen, Z. T., Richards, V. P., Brady, L. J., & Lemos, J. A. (2018). Biology of Oral Streptococci. *Microbiology Spectrum, 6*(5), GPP3-2018. 10.1128/microbiolspec.GPP3-0042-2018

ACNF (2020). *Records of views formed in response to inquiries - Updated 19 November 2020.* https://www.foodstandards.gov.au/industry/novel/novelrecs/pages/default.aspx

Adimpong, D. B., Nielsen, D. S., Sørensen, K. I., Derkx, P. M. F., & Jespersen, L. (2012). Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiology, 12*(1), 75. 10.1186/1471-2180-12-75

Amoroso, P., Avila, F. A., & Gagliardi, C. M. O. (2003). Prevalence of different streptococci species in the oral cavity of children and adolescents. *Brazilian Journal of Oral Sciences, 2*(4), 164-168. 10.20396/bjos.v2i4.8641674

Andaloro, C., Santagati, M., Stefani, S., & La Mantia, I. (2019). Bacteriotherapy with *Streptococcus salivarius* 24SMB and *Streptococcus oralis* 89a oral spray for children with recurrent streptococcal pharyngotonsillitis: a randomized placebo-controlled clinical study. *European Archives of Oto-Rhino-Laryngology, 276*(3), 879-887. 10.1007/s00405-019-05346-3

Ansari, A. S., Dennis, B. B., Shah, D., & Baah, W. (2018). An unusual case of infective pneumocephalus: case report of pneumocephalus exacerbated by continuous positive airway pressure. *BMC Emergency Medicine, 18*(1), 2. 10.1186/s12873-018-0154-9

Aoki-Kinoshita, K., & Kanehisa, M. (2007). Gene Annotation and Pathway Mapping in KEGG. In N. H. Bergman (Ed.), *Comparative Genomics* (pp. 71-91). Humana Press. 10.1007/978-1-59745-515-2_6

Barajas-Colon, E., & Warady, B. A. (2021). *Streptococcus salivarius* peritonitis in an infant receiving chronic peritoneal dialysis. *Perit Dial Int, 41*(3), 341-343. 10.1177/0896860820964285

Barbieri, F., Montanari, C., Gardini, F., & Tabanelli, G. (2019). Biogenic Amine Production by Lactic Acid Bacteria: A Review. *Foods, 8*(1), 17. 10.3390/foods8010017

Barbour, A., Wescombe, P., & Smith, L. (2020). Evolution of Lantibiotic Salivaricins: New Weapons to Fight Infectious Diseases. *Trends in Microbiology, 28*(7), 578-593. 10.1016/j.tim.2020.03.001

Bardellini, E., Amadori, F., Gobbi, E., Ferri, A., Conti, G., & Majorana, A. (2020). Does *Streptococcus salivarius* Strain M18 Assumption Make Black Stains Disappear in Children? *Oral Health & Preventive Dentistry, 18*(2), 161-164. 10.3290/j.ohpd.a43359

Basiotis, P. P., Lino, M., & Dinkins, J. M. (2000). Consumption of Food Group Servings: People's Perceptions vs. Reality. *Nutrition Insights, INSIGHT 20* (USDA Center for Nutrition Policy and Promotion). Available at: https://fns-prod.azureedge.net/sites/default/files/nutrition_insights_uploads/Insight20.pdf

Bellussi, L. M., Villa, M. P., Degiorgi, G., Passali, F. M., Evangelisti, M., Paganelli, I. I., Montesano, M., & Passali, D. (2018). Preventive nasal bacteriotherapy for the treatment of upper respiratory tract infections and sleep disordered breathing in children. *International Journal of Pediatric Otorhinolaryngology, 110*, 43-47. 10.1016/j.ijporl.2018.04.024

Benic, G. Z., Farella, M., Morgan, X. C., Viswam, J., Heng, N. C., Cannon, R. D., & Mei, L. (2019). Oral probiotics reduce halitosis in patients wearing orthodontic braces: a randomized, triple-blind, placebo-controlled trial. *Journal of Breath Research, 13*(3), 036010. 10.1088/1752-7163/ab1c81

Bourdichon, F., Alper, I., Bibiloni, R., Dubois, A., Laulund, S., Miks, M., Morelli, L., Zuliani, V., & Yao, S. (2018). *Inventory of Microbial Food Cultures with safety demonstration in fermented food products. Update of the Bulletin of the IDF 455–2012.* Belgium: International Dairy Federation. Available at: https://store.fil-idf.org/product/bulletin-idf-n-495-2018-inventory-microbial-food-cultures-safety-demonstration-fermented-food-products/

Burton, J. P., Chanyi, R., & Schultz, M. (2017). Chapter 19 - Common Organisms and Probiotics: *Streptococcus thermophilus* (*Streptococcus salivarius* subsp. *thermophilus*). In M. H. Floch, Y. Ringel & W. Allan Walker (Eds.), *The Microbiota in Gastrointestinal Pathophysiology* (pp. 165-169). Academic Press. 10.1016/B978-0-12-804024-9.00019-7

Burton, J. P., Chilcott, C. N., Wescombe, P. A., & Tagg, J. R. (2010). Extended Safety Data for the Oral Cavity Probiotic *Streptococcus salivarius* K12. *Probiotics and Antimicrobial Proteins, 2*(3), 135-144. 10.1007/s12602-010-9045-4

Burton, J. P., Chilcott, C. N., Moore, C. J., Speiser, G., & Tagg, J. R. (2006a). A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. *Journal of Applied Microbiology, 100*(4), 754-764. 10.1111/j.1365-2672.2006.02837.x

Burton, J. P., Cowley, S., Simon, R. R., McKinney, J., Wescombe, P. A., & Tagg, J. R. (2011). Evaluation of safety and human tolerance of the oral probiotic *Streptococcus salivarius* K12: A randomized, placebo-controlled, double-blind study. *Food and Chemical Toxicology, 49*(9), 2356-2364. 10.1016/j.fct.2011.06.038

Burton, J. P., Drummond, B. K., Chilcott, C. N., Tagg, J. R., Thomson, W. M., Hale, J. D. F., & Wescombe, P. A. (2013a). Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. *Journal of Medical Microbiology, 62*(Pt 6), 875-884. 10.1099/jmm.0.056663-0

Burton, J. P., Wescombe, P. A., Macklaim, J. M., Chai, M. H. C., Macdonald, K., Hale, J. D. F., Tagg, J., Reid, G., Gloor, G. B., & Cadieux, P. A. (2013b). Persistence of the oral probiotic *Streptococcus salivarius* M18 is dose dependent and megaplasmid transfer can augment their bacteriocin production and adhesion characteristics. *PloS One, 8*(6), e65991. 10.1371/journal.pone.0065991

Burton, J. P., Wescombe, P. A., Moore, C. J., Chilcott, C. N., & Tagg, J. R. (2006b). Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12. *Applied and Environmental Microbiology, 72*(4), 3050-3053. 10.1128/AEM.72.4.3050-3053.2006

Buxton, R. (2016). *Blood Agar Plates and Hemolysis Protocols. Created 30 September 2005.* American Society for Microbiology. Available at: https://asm.org/Protocols/Blood-Agar-Plates-and-Hemolysis-Protocols

Callon, C., Millet, L., & Montel, M. (2004). Diversity of lactic acid bacteria isolated from AOC Salers cheese. *Journal of Dairy Research, 71*(2), 231-244. 10.1017/S0022029904000159

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics, 10*(1), 421. 10.1186/1471-2105-10-421

Campanella, V., Syed, J., Santacroce, L., Saini, R., Ballini, A., & Inchingolo, F. (2018). Oral probiotics influence oral and respiratory tract infections in pediatric population: a randomized double-blinded placebo-controlled pilot study. *European Review for Medical and Pharmacological Sciences, 22*(22), 8034-8041. 10.26355/eurrev_201811_16433

Cantarutti, A., Rea, F., Donà, D., Cantarutti, L., Passarella, A., Scamarcia, A., Lundin, R., Damiani, V., Giaquinto, C., & Corrao, G. (2020). Preventing recurrent acute otitis media with *Streptococcus salivarius* 24SMB and *Streptococcus oralis* 89a five months intermittent treatment: An observational prospective cohort study. *International Journal of Pediatric Otorhinolaryngology, 132*, 109921. 10.1016/j.ijporl.2020.109921

Chaffanel, F., Charron-Bourgoin, F., Libante, V., Leblond-Bourget, N., & Payot, S. (2015). Resistance Genes and Genetic Elements Associated with Antibiotic Resistance in Clinical and Commensal Isolates of *Streptococcus salivarius*. *Applied and Environmental Microbiology, 81*(12), 4155-4163. 10.1128/AEM.00415-15

Champagne, C. P., Gardner, N. J., & Roy, D. (2005). Challenges in the Addition of Probiotic Cultures to Foods. *Null, 45*(1), 61-84. 10.1080/10408690590900144

Chen, L., Zheng, D., Liu, B., Yang, J., & Jin, Q. (2015). VFDB 2016: hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Research, 44*(D1), D694-D697. 10.1093/nar/gkv1239

Chen, T., Yu, W., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database, 2010*, baq013. 10.1093/database/baq013

Chikindas, M. L., Weeks, R., Drider, D., Chistyakov, V. A., & Dicks, L. M. (2018). Functions and emerging applications of bacteriocins. *Current Opinion in Biotechnology, 49*, 23-28. 10.1016/j.copbio.2017.07.011

CLSI (2020). *Performance standards for antimicrobial susceptibility testing.* Available at: http://em100.edaptivedocs.net/Login.aspx?_ga=2.60916719.252388104.1610393920-271850933.1605299378

Cohen, R., Martin, E., de La Rocque, F., Thollot, F., Pecquet, S., Werner, A., Boucherat, M., Varon, E., Bingen, E., & Levy, C. (2013). Probiotics and Prebiotics in Preventing Episodes of Acute Otitis Media in High-risk Children: A Randomized, Double-blind, Placebo-controlled Study. *The Pediatric Infectious Disease Journal, 32*(8). 10.1097/INF.0b013e31828df4f3

Damaceno, Q. S., Souza, J. P., Nicoli, J. R., Paula, R. L., Assis, G. B., Figueiredo, H. C., Azevedo, V., & Martins, F. S. (2017). Evaluation of Potential Probiotics Isolated from Human Milk and Colostrum. *Probiotics and Antimicrobial Proteins, 9*(4), 371-379. 10.1007/s12602-017-9270-1

De Grandi, R., Bottagisio, M., Di Girolamo, S., Bidossi, A., De Vecchi, E., & Drago, L. (2019a). Modulation of opportunistic species Corynebacterium diphtheriae, Haemophilus parainfluenzae, Moraxella catarrhalis, Prevotella denticola, Prevotella melaninogenica, Rothia dentocariosa, Staphylococcus aureus and Streptococcus pseudopneumoniae by intranasal administration of *Streptococcus salivarius* 24SMBc and *Streptococcus oralis* 89a combination in healthy subjects. *European Review for Medical and Pharmacological Sciences, 23*(1 Suppl), 60-66. 17351 [pii]

De Grandi, R., Drago, L., Bidossi, A., Bottagisio, M., Gelardi, M., & De Vecchi, E. (2019b). Putative Microbial Population Shifts Attributable to Nasal Administration of *Streptococcus salivarius* 24SMBc and *Streptococcus oralis* 89a. *Probiotics and Antimicrobial Proteins, 11*(4), 1219-1226. 10.1007/s12602-018-9488-6

de la Maza, L. M., Pezzlo, M. T., Bittencourt, C. E., & Peterson, E. M. (2020). *Streptococcus*. In *Color Atlas of Medical Bacteriology, Third Edition*. 10.1128/9781683671077.ch2

Delorme, C. (2008). Safety assessment of dairy microorganisms: Streptococcus thermophilus. *International Journal of Food Microbiology, 126*(3), 274-277. 10.1016/j.ijfoodmicro.2007.08.014

Delorme, C., Abraham, A., Renault, P., & Guédon, E. (2015). Genomics of *Streptococcus salivarius*, a major human commensal. *Infection, Genetics and Evolution, 33*, 381-392. 10.1016/j.meegid.2014.10.001

Delorme, C., Poyart, C., Ehrlich, S. D., & Renault, P. (2007). Extent of Horizontal Gene Transfer in Evolution of Streptococci of the Salivarius Group. *Journal of Bacteriology, 189*(4), 1330-1341. 10.1128/JB.01058-06

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of Oral and Maxillofacial Pathology: JOMFP, 23*(1), 122-128. 10.4103/jomfp.JOMFP_304_18

Derrien, M., & van Hylckama Vlieg, J. E. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology, 23*(6), 354-366. S0966-842X(15)00056-6 [pii]

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

Di Pierro, F., Colombo, M., Giuliani, M. G., Danza, M. L., Basile, I., Bollani, T., Conti, A. M., Zanvit, A., & Rottoli, A. S. (2016a). Effect of administration of *Streptococcus salivarius* K12 on the occurrence of

streptococcal pharyngo-tonsillitis, scarlet fever and acute otitis media in 3 years old children. *European Review for Medical and Pharmacological Sciences, 20*(21), 4601-4606. 11696 [pii]

Di Pierro, F., Colombo, M., Zanvit, A., Risso, P., & Rottoli, A. S. (2014). Use of *Streptococcus salivarius* K12 in the prevention of streptococcal and viral pharyngotonsillitis in children. *Drug, Healthcare and Patient Safety, 6*, 15-20. 10.2147/DHPS.S59665

Di Pierro, F., Colombo, M., Zanvit, A., & Rottoli, A. S. (2016b). Positive clinical outcomes derived from using *Streptococcus salivarius* K12 to prevent streptococcal pharyngotonsillitis in children: a pilot investigation. *Drug, Healthcare and Patient Safety, 8*, 77-81. 10.2147/DHPS.S117214

Di Pierro, F., Di Pasquale, D., & Di Cicco, M. (2015a). Oral use of *Streptococcus salivarius* K12 in children with secretory otitis media: preliminary results of a pilot, uncontrolled study. *International Journal of General Medicine, 8*, 303-308. 10.2147/IJGM.S92488

Di Pierro, F., Donato, G., Fomia, F., Adami, T., Careddu, D., Cassandro, C., & Albera, R. (2012). Preliminary pediatric clinical evaluation of the oral probiotic *Streptococcus salivarius* K12 in preventing recurrent pharyngitis and/or tonsillitis caused by Streptococcus pyogenes and recurrent acute otitis media. *International Journal of General Medicine, 5*, 991-997. 10.2147/IJGM.S38859

Di Pierro, F., Risso, P., Poggi, E., Timitilli, A., Bolloli, S., Bruno, M., Caneva, E., Campus, R., & Giannattasio, A. (2018). Use of *Streptococcus salivarius* K12 to reduce the incidence of pharyngo-tonsillitis and acute otitis media in children: a retrospective analysis in not-recurrent pediatric subjects. *Minerva Pediatrica, 70*(3), 240-245. 10.23736/S0026-4946.18.05182-4

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

Dierksen, K. P., Moore, C. J., Inglis, M., Wescombe, P. A., & Tagg, J. R. (2007). The effect of ingestion of milk supplemented with salivaricin A-producing *Streptococcus salivarius* on the bacteriocin-like inhibitory activity of streptococcal populations on the tongue. *FEMS Microbiology Ecology, 59*(3), 584-591. 10.1111/j.1574-6941.2006.00228.x

Dlamini, Z. C., Langa, R. L. S., Aiyegoro, O. A., & Okoh, A. I. (2017). Effects of probiotics on growth performance, blood parameters, and antibody stimulation in piglets. *South African Journal of Animal Science, 47*(6), 766-775. 10.4314/sajas.v47i6.4

Doern, C. D., & Burnham, C. D. (2010). It's not easy being green: the viridans group streptococci, with a focus on pediatric clinical manifestations. *Journal of Clinical Microbiology, 48*(11), 3829-3835. 10.1128/JCM.01563-10

Domínguez-Domínguez, L., de Lagarde-Sebastián, M., de Miguel-Campo, B., & Pulido, F. (2017). Peritonitis bacteriana espontánea por *Streptococcus salivarius* en paciente con coinfección por VIH-VHC en tratamiento con antivirales de acción directa. *Enfermedades Infecciosas Y Microbiología Clínica, 35*(3), 199. 10.1016/j.eimc.2016.02.009

Doyle, H., Pierse, N., Tiatia, R., Williamson, D., Baker, M., & Crane, J. (2018). Effect of Oral Probiotic *Streptococcus salivarius* K12 on Group A Streptococcus Pharyngitis: A Pragmatic Trial in Schools. *The Pediatric Infectious Disease Journal, 37*(7). 10.1097/INF.0000000000001847

EFSA (2006). Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to: The use of nisin (E 234) as a food additive. *EFSA Journal, 4*(3), 314. 10.2903/j.efsa.2006.314

EFSA (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA Journal, 5*(12), 587. 10.2903/j.efsa.2007.587

EFSA BIOHAZ Panel (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal, 9*(10), 2393. 10.2903/j.efsa.2011.2393

EFSA BIOHAZ Panel (2020). The list of QPS status recommended biological agents for safety risk assessments carried out by EFSA [Data set]. *Zenodo,* 10.5281/zenodo.3828466

EFSA FEEDAP (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal, 16*(3), e05206. 10.2903/j.efsa.2018.5206

Facklam, R. (2002). What Happened to the Streptococci: Overview of Taxonomic and Nomenclature Changes. *Clinical Microbiology Reviews, 15*(4), 613. 10.1128/CMR.15.4.613-630.2002

Farrow, J. A., & Collins, M. D. (1984). DNA base composition, DNA-DNA homology and long-chain fatty acid studies on streptococcus thermophilus and *Streptococcus salivarius*. *Journal of General Microbiology, 130*(2), 357-362. 10.1099/00221287-130-2-357

Fields, F. R., Li, X., Navarre, W. W., & Naito, M. (2020). Complete Genome Sequence of *Streptococcus salivarius* DB-B5, a Novel Probiotic Candidate Isolated from the Supragingival Plaque of a Healthy Female Subject. *Microbiol Resour Announc, 9*(40), 916. 10.1128/MRA.00916-20

Fléchard, M., Lucchetti-Miganeh, C., Hallet, B., Hols, P., & Gilot, P. (2019). Intensive targeting of regulatory competence genes by transposable elements in streptococci. *Molecular Genetics and Genomics, 294*(3), 531-548. 10.1007/s00438-018-1507-5

Freire, A. L., Zapata, S., Mosquera, J., Mejia, M. L., & Trueba, G. (2016). Bacteria associated with human saliva are major microbial components of Ecuadorian indigenous beers (chicha). *PeerJ (San Francisco, CA), 4*, e1962. 10.7717/peerj.1962

Gilbey, P., Livshits, L., Sharabi-Nov, A., Avraham, Y., & Miron, D. (2015). Probiotics in addition to antibiotics for the treatment of acute tonsillitis: a randomized, placebo-controlled study. *European Journal of Clinical Microbiology & Infectious Diseases, 34*(5), 1011-1015. 10.1007/s10096-015-2315-z

Goldstein, E. J. C., Tyrrell, K. L., & Citron, D. M. (2015). Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. *Clinical Infectious Diseases, 60*(suppl_2), S98-S107. 10.1093/cid/civ072

Gregori, G., Righi, O., Risso, P., Boiardi, G., Demuru, G., Ferzetti, A., Galli, A., Ghisoni, M., Lenzini, S., Marenghi, C., Mura, C., Sacchetti, R., & Suzzani, L. (2016). Reduction of group A beta-hemolytic streptococcus pharyngo-tonsillar infections associated with use of the oral probiotic *Streptococcus salivarius* K12: a retrospective observational study. *Therapeutics and Clinical Risk Management, 12*, 87-92. 10.2147/TCRM.S96134

Hall, J. E. (2011). Secretory Functions of the Alimentary Tract - Chapter 64. *Guyton and Hall Textbook of Medical Physiology - Twelfth Edition* (pp. 773-788). Elselvier.

Hamada, S., Ooshima, T., Torii, M., Imanishi, H., Masuda, N., Sobue, S., & Kotani, S. (1978). Dental Caries Induction in Experimental Animals by Clinical Strains of Streptococcus mutans Isolated from Japanese Children. *Microbiology and Immunology, 22*(6), 301-314. 10.1111/j.1348-0421.1978.tb00375.x

Han, C. S., Martin, M. A., Dichosa, A. E. K., Daughton, A. R., Frietze, S., Kaplan, H., Gurven, M. D., & Alcock, J. (2016). Salivary microbiomes of indigenous Tsimane mothers and infants are distinct despite frequent premastication. *PeerJ, 4*, e2660. 10.7717/peerj.2660

He, L., Yang, H., Chen, Z., & Ouyang, X. (2020). The Effect of *Streptococcus salivarius* K12 on Halitosis: a Double-Blind, Randomized, Placebo-Controlled Trial. *Probiotics and Antimicrobial Proteins*, 10.1007/s12602-020-09646-7

Heng, N. C. K., Haji-Ishak, N., Kalyan, A., Wong, A. Y. C., Lovrić, M., Bridson, J. M., Artamonova, J., Stanton, J. L., Wescombe, P. A., Burton, J. P., Cullinan, M. P., & Tagg, J. R. (2011). Genome Sequence of the Bacteriocin-Producing Oral Probiotic *Streptococcus salivarius* Strain M18. *Journal of Bacteriology, 193*(22), 6402. 10.1128/JB.06001-11

Heng, N. C. K., & Tagg, J. R. (2006). What's in a name? Class distinction for bacteriocins. *Nature Reviews Microbiology, 4*(2), 160. 10.1038/nrmicro1273-c1

Hesselmar, B., Sjöberg, F., Saalman, R., Åberg, N., Adlerberth, I., & Wold, A. E. (2013). Pacifier Cleaning Practices and Risk of Allergy Development. *Pediatrics, 131*(6), e1829-e1837. 10.1542/peds.2012-3345

Hevroni, G., Maryniak, A., Cepeda-Mora, D., Salifu, M. O., & McFarlane, S. I. (2020). Paravalvular Abscess as a Complication of *Streptococcus salivarius* Infective Endocarditis of a Bioprosthetic Aortic Valve. *American Journal of Medical Case Reports, 8*(11), 405-408. 10.12691/ajmcr-8-11-7

Hols, P., Ledesma-García, L., Gabant, P., & Mignolet, J. (2019). Mobilization of Microbiota Commensals and Their Bacteriocins for Therapeutics. *Trends in Microbiology, 27*(8), 690-702. 10.1016/j.tim.2019.03.007

Horz, H. P., Meinelt, A., Houben, B., & Conrads, G. (2007). Distribution and persistence of probiotic *Streptococcus salivarius* K12 in the human oral cavity as determined by real-time quantitative polymerase chain reaction. *Oral Microbiology and Immunology, 22*(2), 126-130. 10.1111/j.1399-302X.2007.00334.x

Hu, L., Mao, Q., Zhou, P., Lv, X., Hua, H., & Yan, Z. (2019). Effects of *Streptococcus salivarius* K12 with nystatin on oral candidiasis—RCT. *Oral Diseases, 25*(6), 1573-1580. 10.1111/odi.13142

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature, 486*(7402), 207-214. 10.1038/nature11234

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: Normal composition, flow, and function. *The Journal of Prosthetic Dentistry, 85*(2), 162-169. 10.1067/mpr.2001.113778

Hyink, O., Wescombe, P. A., Upton, M., Ragland, N., Burton, J. P., & Tagg, J. R. (2007). Salivaricin A2 and the Novel Lantibiotic Salivaricin B Are Encoded at Adjacent Loci on a 190-Kilobase Transmissible Megaplasmid in the Oral Probiotic Strain *Streptococcus salivarius* K12. *Applied and Environmental Microbiology, 73*(4), 1107-1113. 10.1128/AEM.02265-06

Iorgulescu, G. (2009). Saliva between normal and pathological. Important factors in determining systemic and oral health. *Journal of Medicine and Life, 2*(3), 303-307.

Ishijima, S. A., Hayama, K., Burton, J. P., Reid, G., Okada, M., Matsushita, Y., & Abe, S. (2012). Effect of *Streptococcus salivarius* K12 on the In Vitro Growth of Candida albicans and Its Protective Effect in an Oral Candidiasis Model. *Applied and Environmental Microbiology, 78*(7), 2190-2199. 10.1128/AEM.07055-11

ITIS (2012). *Streptococcus thermophilus Orla-Jensen, 1919 - Taxonomic Serial No.: 966477.* Available at: http://www.itis.gov.

Jamali, Z., Aminabadi, N. A., Samiei, M., Sighari Deljavan, A., Shokravi, M., & Shirazi, S. (2016). Impact of Chlorhexidine Pretreatment Followed by Probiotic *Streptococcus salivarius* Strain K12 on Halitosis in Children: A Randomised Controlled Clinical Trial. *Oral Health & Preventive Dentistry, 14*(4), 305-313. 10.3290/j.ohpd.a36521

Jans, C., Meile, L., Kaindi, D. W. M., Kogi-Makau, W., Lamuka, P., Renault, P., Kreikemeyer, B., Lacroix, C., Hattendorf, J., Zinsstag, J., Schelling, E., Fokou, G., & Bonfoh, B. (2017). African fermented dairy products – Overview of predominant technologically important microorganisms focusing on African Streptococcus infantarius variants and potential future applications for enhanced food safety and security. *International Journal of Food Microbiology, 250*, 27-36. 10.1016/j.ijfoodmicro.2017.03.012

Jenks, K., Stebbings, S., Burton, J., Schultz, M., Herbison, P., & Highton, J. (2010). Probiotic Therapy for the Treatment of Spondyloarthritis: A Randomized Controlled Trial. *The Journal of Rheumatology, 37*(10), 2118-2125. 10.3899/jrheum.100193

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., Lago, B. A., Dave, B. M., Pereira, S., Sharma, A. N., Doshi, S., Courtot, M., Lo, R., Williams, L. E., Frye, J. G., Elsayegh, T., Sardar, D.,

Westman, E. L., Pawlowski, A. C., . . . McArthur, A. G. (2017). CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Research, 45*(D1), D566-D573. 10.1093/nar/gkw1004

Johansson, M. H. K., Bortolaia, V., Tansirichaiya, S., Aarestrup, F. M., Roberts, A. P., & Petersen, T. N. (2020). Detection of mobile genetic elements associated with antibiotic resistance in Salmonella enterica using a newly developed web tool: MobileElementFinder. *Journal of Antimicrobial Chemotherapy, 76*(1), 101-109. 10.1093/jac/dkaa390

Jovanovic, U., Freyer, M., & Heckmann, J. G. (2019). *Streptococcus salivarius* meningitis: a spontaneous case in a 74-year-old man. *Acta Neurologica Belgica, 119*(3), 481-482. 10.1007/s13760-018-0891-2

Jun, J. H. (2019). Simultaneous Triple Microbial Keratitis. *Korean Journal of Ophthalmology : KJO, 33*(6), 573-574. 10.3341/kjo.2019.0032

Kadri, Z., Spitaels, F., Cnockaert, M., Amar, M., Joossens, M., & Vandamme, P. (2021). The bacterial diversity of raw Moroccon camel milk. *International Journal of Food Microbiology, 341*, 109050. 10.1016/j.ijfoodmicro.2021.109050

Kanehisa, M. (2018). Inferring Antimicrobial Resistance from Pathogen Genomes in KEGG. In H. Mamitsuka (Ed.), *Data Mining for Systems Biology: Methods and Protocols* (pp. 225-239). Springer New York. 10.1007/978-1-4939-8561-6_17

Kanehisa, M., Sato, Y., & Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *Journal of Molecular Biology, 428*(4), 726-731. 10.1016/j.jmb.2015.11.006

Kaškonienė, V., Stankevičius, M., Bimbiraitė-Survilienė, K., Naujokaitytė, G., Šernienė, L., Mulkytė, K., Malakauskas, M., & Maruška, A. (2017). Current state of purification, isolation and analysis of bacteriocins produced by lactic acid bacteria. *Applied Microbiology and Biotechnology, 101*(4), 1323-1335. 10.1007/s00253-017-8088-9

Khalesi, S., Bellissimo, N., Vandelanotte, C., Williams, S., Stanley, D., & Irwin, C. (2018). A review of probiotic supplementation in healthy adults: helpful or hype? *European Journal of Clinical Nutrition, 73*(1), 24-37. 10.1038/s41430-018-0135-9

Kilian, M., Chapple, I. L. C., Hannig, M., Marsh, P. D., Meuric, V., Pedersen, A. M. L., Tonetti, M. S., Wade, W. G., & Zaura, E. (2016). The oral microbiome – an update for oral healthcare professionals. *British Dental Journal, 221*(10), 657-666. 10.1038/sj.bdj.2016.865

Kort, R., Caspers, M., van de Graaf, A., van Egmond, W., Keijser, B., & Roeselers, G. (2014). Shaping the oral microbiota through intimate kissing. *Microbiome, 2*(1), 41. 10.1186/2049-2618-2-41

Krittaphol, W., Wescombe, P. A., Thomson, C. D., McDowell, A., Tagg, J. R., & Fawcett, J. P. (2011). Metabolism of l-Selenomethionine and Selenite by Probiotic Bacteria: In Vitro and In Vivo Studies. *Biological Trace Element Research, 144*(1), 1358-1369. 10.1007/s12011-011-9057-2

La Mantia, I., Varricchio, A., & Ciprandi, G. (2017). Bacteriotherapy with *Streptococcus salivarius* 24SMB and *Streptococcus oralis* 89a nasal spray for preventing recurrent acute otitis media in children: a real-life clinical experience. *International Journal of General Medicine, 10*, 171-175. 10.2147/IJGM.S137614

Lancefield, R. C. (1933). A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci. *The Journal of Experimental Medicine, 57*(4), 571-595. 10.1084/jem.57.4.571

Lang, J. M., Eisen, J. A., & Zivkovic, A. M. (2014). The microbes we eat: abundance and taxonomy of microbes consumed in a day's worth of meals for three diet types. *PeerJ, 2*, e659. 10.7717/peerj.659

Lechner, A. M., Pretsch, I., Hoppe, U., Seitelberger, R., & Dinges, C. (2020). Successful long-term antibiotic suppressive therapy in a case of prosthetic valve endocarditis and a case of extensive aortic and subclavian graft infection. *Infection, 48*(1), 133-136. 10.1007/s15010-019-01321-6

Lee, H. J., Orlovich, D. A., Tagg, J. R., & Fawcett, J. P. (2009). Detection and Specific Enumeration of Multi-Strain Probiotics in the Lumen Contents and Mucus Layers of the Rat Intestine After Oral Administration. *Probiotics and Antimicrobial Proteins, 1*(2), 113-120. 10.1007/s12602-009-9019-6

Lee, H. J., Waller, R. D., Stebbings, S., Highton, J., Orlovich, D. A., Schmierer, D., & Fawcett, J. P. (2010). The effects of an orally administered probiotic on sulfasalazine metabolism in individuals with rheumatoid arthritis: a preliminary study. *International Journal of Rheumatic Diseases, 13*(1), 48-54. 10.1111/j.1756-185X.2009.01449.x

Lee, H. J., Zhang, H., Orlovich, D. A., & Fawcett, J. P. (2012). The influence of probiotic treatment on sulfasalazine metabolism in rat. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems, 42*(8), 791-797. 10.3109/00498254.2012.660508

Li, X., Fields, F. R., Ho, M., Marshall-Hudson, A., Gross, R., Casser, M. E., & Naito, M. (2021). Safety assessment of *Streptococcus salivarius* DB-B5 as a probiotic candidate for oral health. *Food and Chemical Toxicology, 153*, 112277. 10.1016/j.fct.2021.112277

Li, Y., Shao, F., Zheng, S., Tan, Z., & He, Y. (2020). Alteration of *Streptococcus salivarius* in Buccal Mucosa of Oral Lichen Planus and Controlled Clinical Trial in OLP Treatment. *Probiotics and Antimicrobial Proteins,* 10.1007/s12602-020-09664-5

Loesche, W. J., Schork, A., Terpenning, M. S., Chen, Y. M., & Stoll, J. (1995). Factors which influence levels of selected organisms in saliva of older individuals. *Journal of Clinical Microbiology, 33*(10), 2550-2557. http://jcm.asm.org/content/33/10/2550.abstract

López-Cuellar, M. d. R., Rodríguez-Hernández, A., & Chavarría-Hernández, N. (2016). LAB bacteriocin applications in the last decade. *Null, 30*(6), 1039-1050. 10.1080/13102818.2016.1232605

Manti, S., Parisi, G. F., Papale, M., Licari, A., Salpietro, C., Miraglia Del Giudice, M., Marseglia, G. L., & Leonardi, S. (2020). Bacteriotherapy with *Streptococcus salivarius* 24SMB and *Streptococcus oralis*

89a nasal spray for treatment of upper respiratory tract infections in children: a pilot study on short-term efficacy. *Italian Journal of Pediatrics, 46*(1), 42. 10.1186/s13052-020-0798-4

Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E. (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. *International Dairy Journal, 16*(3), 189-199. 10.1016/j.idairyj.2005.02.009

Marchisio, P., Santagati, M., Scillato, M., Baggi, E., Fattizzo, M., Rosazza, C., Stefani, S., Esposito, S., & Principi, N. (2015). *Streptococcus salivarius* 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. *European Journal of Clinical Microbiology & Infectious Diseases, 34*(12), 2377-2383. 10.1007/s10096-015-2491-x

Marco, M. L., Hill, C., Hutkins, R., Slavin, J., Tancredi, D. J., Merenstein, D., & Sanders, M. E. (2020). Should There Be a Recommended Daily Intake of Microbes? *The Journal of Nutrition, 150*(12), 3061–3067. 10.1093/jn/nxaa323

Marini, G., Sitzia, E., Panatta, M. L., & De Vincentiis, G. C. (2019). Pilot study to explore the prophylactic efficacy of oral probiotic *Streptococcus salivarius* K12 in preventing recurrent pharyngo-tonsillar episodes in pediatric patients. *International Journal of General Medicine, 12*, 213-217. 10.2147/IJGM.S168209

Marshall, V. M., Cole, W. M., & Phillips, B. A. (1985). Fermentation of milk by *Streptococcus salivarius* subspecies salivarius and *Streptococcus salivarius* subspecies thermophilus and their use to the yoghurt manufacturer. *Journal of Applied Bacteriology, 59*(2), 147-151. 10.1111/j.1365-2672.1985.tb03314.x

Martinović, A., Cocuzzi, R., Arioli, S., & Mora, D. (2020). Streptococcus thermophilus: To Survive, or Not to Survive the Gastrointestinal Tract, That Is the Question! *Nutrients, 12*(8), 2175.

Mehanna, C., Kallassi, L., Mansour, A. M., & Hamam, R. N. (2021). *Streptococcus salivarius* endogenous endophthalmitis. *BMJ Case Reports, 14*(2), e239299. 10.1136/bcr-2020-239299

Mignolet, J., Fontaine, L., Sass, A., Nannan, C., Mahillon, J., Coenye, T., & Hols, P. (2018). Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*. *Cell Reports, 22*(7), 1627-1638. 10.1016/j.celrep.2018.01.055

Moradi, M., Kousheh, S. A., Almasi, H., Alizadeh, A., Guimarães, J. T., Yılmaz, N., & Lotfi, A. (2020). Postbiotics produced by lactic acid bacteria: The next frontier in food safety. *Comprehensive Reviews in Food Science and Food Safety, n/a*10.1111/1541-4337.12613

Motato, K. E., Milani, C., Ventura, M., Valencia, F. E., Ruas-Madiedo, P., & Delgado, S. (2017). Bacterial diversity of the Colombian fermented milk "Suero Costeño" assessed by culturing and high-throughput sequencing and DGGE analysis of 16S rRNA gene amplicons. *Food Microbiology, 68*, 129-136. 10.1016/j.fm.2017.07.011

Nobbs, A. H., Lamont, R. J., & Jenkinson, H. F. (2009). Streptococcus adherence and colonization. *Microbiology and Molecular Biology Reviews, 73*(3), 407-450. 10.1128/MMBR.00014-09

Oblitas, C. M., Sánchez-Soblechero, A., & Pulfer, M. D. (2020). "Sweet tears": *Streptococcus salivarius* meningitis secondary to ethmoidal mucocele. *Neurología (English Edition), 35*(9), 687-690. 10.1016/j.nrleng.2019.10.003

Obodai, M., & Dodd, C. E. R. (2006). Characterization of dominant microbiota of a Ghanaian fermented milk product, nyarmie, by culture- and nonculture-based methods. *Journal of Applied Microbiology, 100*(6), 1355-1363. 10.1111/j.1365-2672.2006.02895.x

Olson, L. B., Turner, D. J., Cox, G. M., & Hostler, C. J. (2019). *Streptococcus salivarius* Prosthetic Joint Infection following Dental Cleaning despite Antibiotic Prophylaxis. *Case Reports in Infectious Diseases, 2019*, 8109280. 10.1155/2019/8109280

Ongol, M. P., & Asano, K. (2009). Main microorganisms involved in the fermentation of Ugandan ghee. *International Journal of Food Microbiology, 133*(3), 286-291. 10.1016/j.ijfoodmicro.2009.06.003

Özogul, Y., & Özogul, F. (2019). Biogenic Amines Formation, Toxicity, Regulations in Food. In B. Saad, & R. Tofalo (Eds.), *Chapter 1: Biogenic Amines in Food: Analysis, Occurrence and Toxicity* (pp. 1-17). The Royal Society of Chemistry. 10.1039/9781788015813-00001

Pariza, M. W., Gillies, K. O., Kraak-Ripple, S. F., Leyer, G., & Smith, A. B. (2015). Determining the safety of microbial cultures for consumption by humans and animals. *Regulatory Toxicology and Pharmacology, 73*(1), 164-171. 10.1016/j.yrtph.2015.07.003

Parks, T., Barrett, L., & Jones, N. (2015). Invasive streptococcal disease: a review for clinicians. *British Medical Bulletin, 115*(1), 77-89. 10.1093/bmb/ldv027

Passali, D., Passali, G. C., Vesperini, E., Cocca, S., Visconti, I. C., Ralli, M., & Bellussi, L. M. (2019). The efficacy and tolerability of *Streptococcus salivarius* 24SMB and *Streptococcus oralis* 89a administered as nasal spray in the treatment of recurrent upper respiratory tract infections in children. *European Review for Medical and Pharmacological Sciences, 23*(1 Suppl), 67-72. 17352 [pii]

Passariello, C., Di Nardo, F., Polimeni, A., Di Nardo, D., & Testarelli, L. (2020). *Probiotic Streptococcus salivarius Reduces Symptoms of Denture Stomatitis and Oral Colonization by Candida albicans*10.3390/app10093002

Patras, K. A., Wescombe, P. A., Rösler, B., Hale, J. D., Tagg, J. R., & Doran, K. S. (2015). *Streptococcus salivarius* K12 Limits Group B Streptococcus Vaginal Colonization. *Infection and Immunity, 83*(9), 3438-3444. 10.1128/IAI.00409-15

Patterson, M. J. (1996). Streptococcus. In S. Baron (Ed.), *Medical Microbiology. 4th edition* (pp. Chapter 13.). University of Texas Medical Branch at Galveston.

Pešić-Mikulec, D., & Jovanović, L. (2006). Microbiological study of fresh white cheese (a Serbian craft variety). *Applied Ecology and Environmental Research, 4*(1), 129-134. 10.15666/aeer/0401_129134

Pombert, J., Sistek, V., Boissinot, M., & Frenette, M. (2009). Evolutionary relationships among salivarius streptococci as inferred from multilocus phylogenies based on 16S rRNA-encoding, recA, secA, and secY gene sequences. *BMC Microbiology, 9*(1), 232. 10.1186/1471-2180-9-232

Power, D., Burton, J., Chilcott, C., Dawes, P., & Tagg, J. (2008). Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral probiotic *Streptococcus salivarius* K12. *European Journal of Clinical Microbiology & Infectious Diseases, 27*(12), 1261-1263. 10.1007/s10096-008-0569-4

Pradhan, D., Mallappa, R. H., & Grover, S. (2020). Comprehensive approaches for assessing the safety of probiotic bacteria. *Food Control, 108*, 106872. 10.1016/j.foodcont.2019.106872

Public Health Agency of Canada. (2018). *Pathogen Safety Data Sheet: Infectious Substances – Streptococcus salivarius* . Canada: Public Health Agency of Canada. Available at: https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/streptococcus-salivarius.html

Rezac, S., Kok, C. R., Heermann, M., & Hutkins, R. (2018). Fermented Foods as a Dietary Source of Live Organisms. *Frontiers in Microbiology, 9*, 1785. 10.3389/fmicb.2018.01785

Riane, K., Sifour, M., Ouled-Haddar, H., Espinosa, C., Esteban, M. A., & Lahouel, M. (2020). Effect of probiotic supplementation on oxidative stress markers in rats with diclofenac-induced hepatotoxicity. *Brazilian Journal of Microbiology,* 10.1007/s42770-020-00302-4

Ruiz, L., Bacigalupe, R., García-Carral, C., Boix-Amoros, A., Argüello, H., Silva, C. B., de los Angeles Checa, Maria, Mira, A., & Rodríguez, J. M. (2019). Microbiota of human precolostrum and its potential role as a source of bacteria to the infant mouth. *Scientific Reports, 9*(1), 8435. 10.1038/s41598-019-42514-1

Şanlier, N., Gökcen, B. B., & Sezgin, A. C. (2019). Health benefits of fermented foods. *Critical Reviews in Food Science and Nutrition, 59*(3), 506-527. 10.1080/10408398.2017.1383355

Santagati, M., Scillato, M., Muscaridola, N., Metoldo, V., La Mantia, I., & Stefani, S. (2015). Colonization, safety, and tolerability study of the *Streptococcus salivarius* 24SMBc nasal spray for its application in upper respiratory tract infections. *European Journal of Clinical Microbiology & Infectious Diseases, 34*(10), 2075-2080. 10.1007/s10096-015-2454-2

Sarlin, S., Tejesvi, M. V., Turunen, J., Vänni, P., Pokka, T., Renko, M., & Tapiainen, T. (2021). Impact of *Streptococcus salivarius* K12 on Nasopharyngeal and Saliva Microbiome: A Randomized Controlled Trial. *The Pediatric Infectious Disease Journal, 40*(5). 10.1097/INF.0000000000003016

Schleifer, K. H., Ehrmann, M., Krusch, U., & Neve, H. (1991). Revival of the Species Streptococcus thermophilus (ex Orla-Jensen, 1919) nom. rev. *Systematic and Applied Microbiology, 14*(4), 386-388. 10.1016/S0723-2020(11)80314-0

Shane, A. L., Cabana, M. D., Ellis, C. L., Heimbach, J. T., Hempel, S., Hummelen, R., Lynch, S., Merenstein, D. J., Sanders, M. E., Tancredi, D. J., & Vidry, S. (2010). Guide to designing, conducting, publishing,

and communicating results of clinical studies involving probiotic applications in human participants. *Gut Microbes, 1*(4), 243-253. 10.4161/gmic.1.4.12707

Sherman, J. M. (1937). THE STREPTOCOCCI. *Bacteriological Reviews, 1*(1), 3. http://mmbr.asm.org/content/1/1/3.abstract

Siegrist, J. (Unknown). Streptococci - Overview of Detection, Identification, Differentiation and Cultivation Techniques. *AnalytiX, 7*. Available at: https://www.sigmaaldrich.com/technical-documents/articles/analytix/streptococci-overview.html

Silva, D. R., Sardi, Janaína de Cássia Orlandi, Pitangui, N. d. S., Roque, S. M., Silva, Andréa Cristina Barbosa da, & Rosalen, P. L. (2020). Probiotics as an alternative antimicrobial therapy: Current reality and future directions. *Journal of Functional Foods, 73*, 104080. 10.1016/j.jff.2020.104080

Sitkiewicz, I., & Hryniewicz, W. (2010). Pyogenic streptococci–danger of re-emerging pathogens. *Polish Journal of Microbiology, 59*(4), 219-226.

Soltani, S., Hammami, R., Cotter, P. D., Rebuffat, S., Said, L. B., Gaudreau, H., Bédard, F., Biron, E., Drider, D., & Fliss, I. (2020). Bacteriocins as a new generation of antimicrobials: Toxicity aspects and regulations. *FEMS Microbiology Reviews, 45*(1), fuaa039. 10.1093/femsre/fuaa039

Tamang, J. P., Shin, D., Jung, S., & Chae, S. (2016). Functional Properties of Microorganisms in Fermented Foods. *Frontiers in Microbiology, 7*, 578. 10.3389/fmicb.2016.00578

Tanzer, J. M., Kurasz, A. B., & Clive, J. (1985). Competitive displacement of mutans streptococci and inhibition of tooth decay by *Streptococcus salivarius* TOVE-R. *Infection and Immunity, 48*(1), 44. 10.1128/iai.48.1.44-50.1985

Tarantino, V., Savaia, V., D'Agostino, R., Damiani, V., & Ciprandi, G. (2020). Oral bacteriotherapy in children with recurrent respiratory infections: a real-life study. *Acta Bio-Medica : Atenei Parmensis, 91*(1-S), 73-76. 10.23750/abm.v91i1-S.9230

Tarantino, V., Savaia, V., D'Agostino, R., Silvestri, M., & Ciprandi, G. (2018). Bacteriotherapy for preventing recurrent upper respiratory infections in children: a real-world experience. *Otolaryngologia Polska the Polish Otolaryngology, 72*(3), 33-38. 10.5604/01.3001.0012.0482

Tarantino, V., Savaia, V., D'Agostino, R., Silvestri, M., Passali, F. M., Di Girolamo, S., & Ciprandi, G. (2019). Bacteriotherapy in children with recurrent upper respiratory tract infections. *European Review for Medical and Pharmacological Sciences, 23*(1 Suppl), 39-43. 17347 [pii]

TGA (2019). *Compositional guideline: Streptococcus salivarius M18.* Australian Government, Department of Health. Available at: https://www.tga.gov.au/compositional-guideline/streptococcus-salivarius-m18

Uriot, O., Denis, S., Junjua, M., Roussel, Y., Dary-Mourot, A., & Blanquet-Diot, S. (2017). Streptococcus thermophilus: From yogurt starter to a new promising probiotic candidate? *Journal of Functional Foods, 37*, 74-89. 10.1016/j.jff.2017.07.038

Van Hoorde, K., Verstraete, T., Vandamme, P., & Huys, G. (2008). Diversity of lactic acid bacteria in two Flemish artisan raw milk Gouda-type cheeses. *Food Microbiology, 25*(7), 929-935. 10.1016/j.fm.2008.06.006

Vargas Osorio, M. P., Muñoz Montoya, J. E., Charry Lopez, M. L., & Rojas Romero, L. O. (2019). Meningitis for *Streptococcus salivarius* Secondary to Paradoxical Cerebrospinal Fluid Rhinorrhea as a Complication of Retrosigmoid Approach. *Asian Journal of Neurosurgery, 14*(1), 310-313. 10.4103/ajns.AJNS_179_18

Vesty, A., Gear, K., Boutell, S., Taylor, M. W., Douglas, R. G., & Biswas, K. (2020). Randomised, double-blind, placebo-controlled trial of oral probiotic *Streptococcus salivarius* M18 on head and neck cancer patients post-radiotherapy: a pilot study. *Scientific Reports, 10*(1), 13201. 10.1038/s41598-020-70024-y

Wang, C. Y., & Dawid, S. (2018). Mobilization of Bacteriocins during Competence in Streptococci. *Trends in Microbiology, 26*(5), 389-391. 10.1016/j.tim.2018.03.002

Watanabe, S., Ohnishi, M., Imai, K., Kawano, E., & Igarashi, S. (1995). Estimation of the total saliva volume produced per day in five-year-old children. *Archives of Oral Biology, 40*(8), 781-782. https://doi.org/10.1016/0003-9969(95)00026-L

Wescombe, P. A., Hale, J. D. F., Heng, N. C. K., & Tagg, J. R. (2012). Developing oral probiotics from *Streptococcus salivarius*. *Future Microbiology, 7*(12), 1355-1371. 10.2217/fmb.12.113

Wescombe, P. A., Heng, N. C. K., Burton, J. P., Chilcott, C. N., & Tagg, J. R. (2009). Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. *Future Microbiology, 4*(7), 819-835. 10.2217/fmb.09.61

Wescombe, P., Burton, J., Cadieux, P., Klesse, N., Hyink, O., Heng, N., Chilcott, C., Reid, G., & Tagg, J. (2006). Megaplasmids encode differing combinations of lantibiotics in *Streptococcus salivarius*. *Antonie Van Leeuwenhoek, 90*(3), 269-280. 10.1007/s10482-006-9081-y

Whiley, R. A., & Hardie, J. M. (2015). *Streptococcus*. John Wiley & Sons, Inc. 10.1002/9781118960608.gbm00612

WHO/FAO (2009). *Codex Alimentarius - Foods Derived from Modern Biotechnology, 2nd edition*. World Health Organization. (WHO) / Food and Agriculture Organization of the United Nations (FAO).

WHO/FAO (2018). *Standard for Fermented Milks. CODEX STAN 243-2003. Adopted in 2003. Revised in 2008, 2010, 2018*. Rome: FAO.

Wu, D., Hugenholtz, P., Mavromatis, K., Pukall, R., Dalin, E., Ivanova, N. N., Kunin, V., Goodwin, L., Wu, M., Tindall, B. J., Hooper, S. D., Pati, A., Lykidis, A., Spring, S., Anderson, I. J., D'haeseleer, P., Zemla, A., Singer, M., Lapidus, A., . . . Eisen, J. A. (2009). A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. *Nature, 462*(7276), 1056-1060. 10.1038/nature08656

Xiao, J., Fiscella, K. A., & Gill, S. R. (2020). Oral microbiome: possible harbinger for children's health. *International Journal of Oral Science, 12*(1), 12. 10.1038/s41368-020-0082-x

Yang, S., Lin, C., Sung, C. T., & Fang, J. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology, 5*, 241. 10.3389/fmicb.2014.00241

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
   ☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____
2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*
3. Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2021-06-01
4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Mizue Naito	Director, Probiotics & Microbiome R&D

Organization *(if applicable)*: Dose Biosystems Inc.

Mailing Address *(number and street)*: 661 University Ave, Suite 1300

City	State or Province	Zip Code/Postal Code	Country
Toronto	Ontario	M5G 0B7	Canada

Telephone Number	Fax Number	E-Mail Address
1-416-986-3750		mizue@dosebiosystems.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title

Organization *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Streptococcus salivarius DB-B5

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway ☐ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 1

Total number of pages 69

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 807

☐ b) GRAS Affirmation Petition No. GRP

☐ c) Food Additive Petition No. FAP

☐ d) Food Master File No. FMF

☒ e) Other or Additional *(describe or enter information as above)* GRN No. 591 is also incorporated by reference.

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8) and 170.250(d) and (e))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Dose Biosystems intends to use *S. salivarius* DB-B5 as a general ingredient in conventional foods at target levels providing a minimum of $1x10^9$ CFU/serving. *S. salivarius* DB-B5 is not intended for addition to infant formula, or to meat and poultry products that are subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA).

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Dose Biosystems Inc.
(name of notifier)

has concluded that the intended use*(s)* of Streptococcus salivarius DB-B5
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Dose Biosystems Inc. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them;
(name of notifier)

agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

661 University Ave, Suite 1300, Toronto, ON M5G 0B7, Canada
(address of notifier or other location)

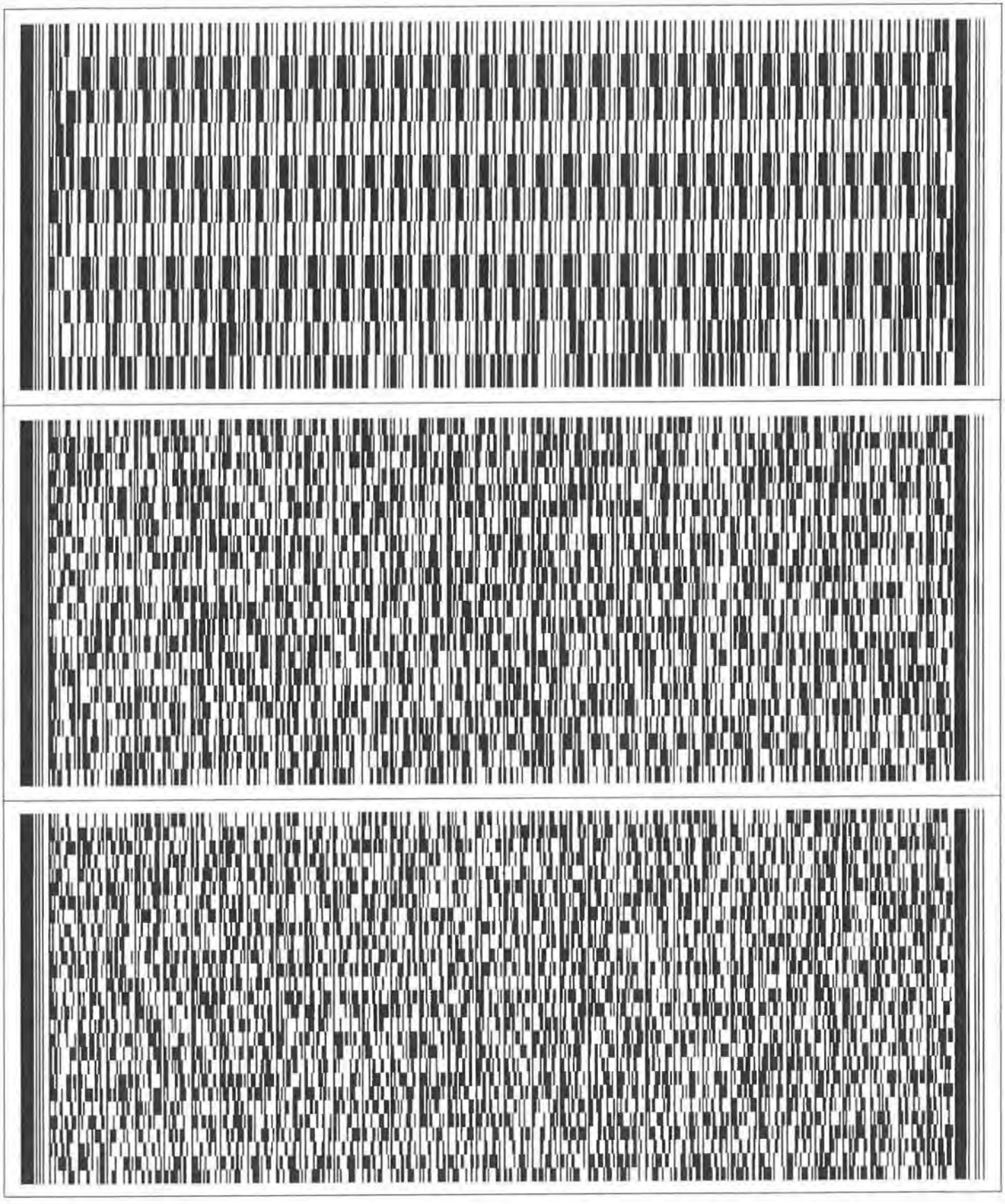
The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

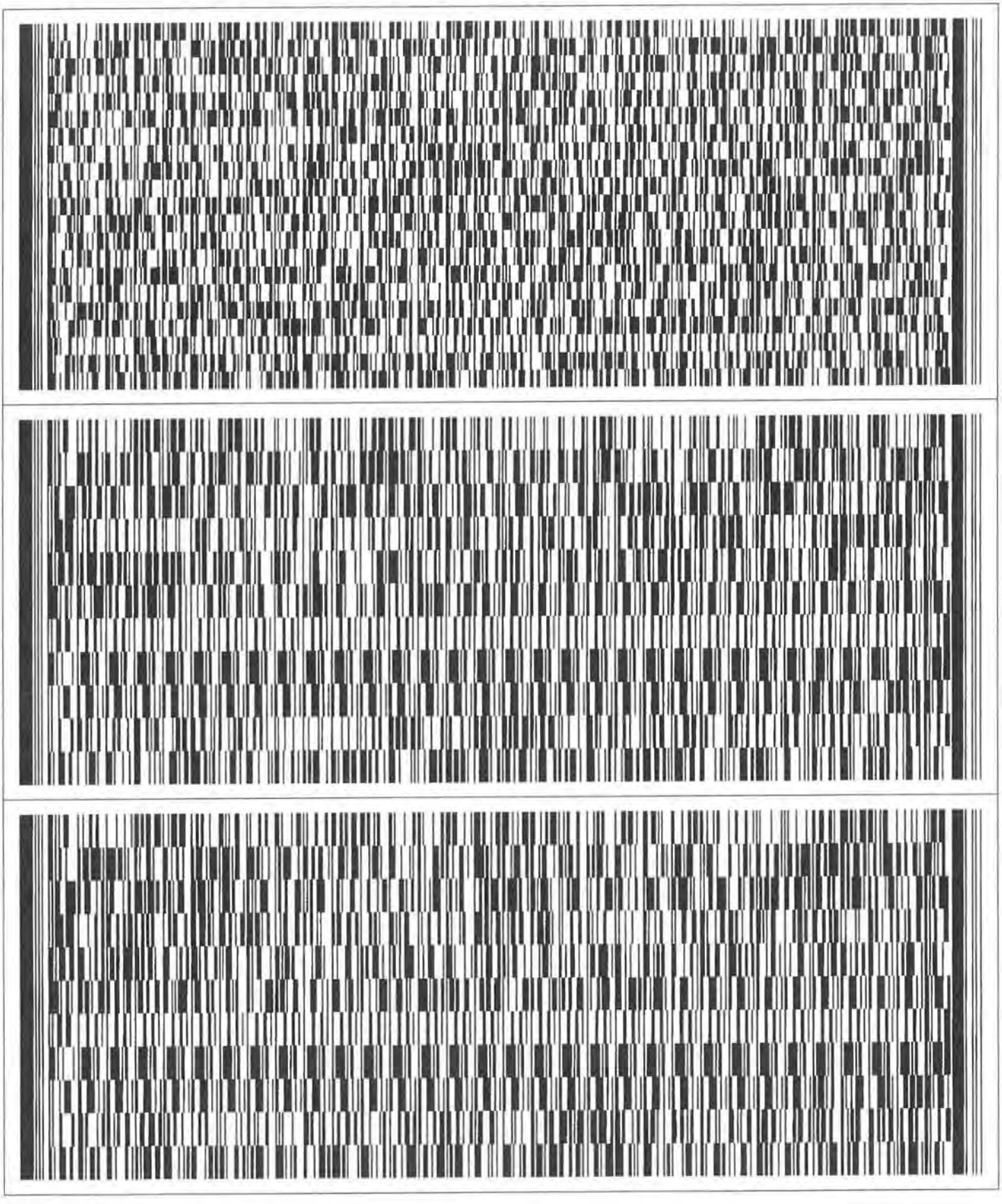
3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
[redacted]	Mizue Naito, Director, Probiotics & Microbiome R&D	06/07/2021

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)





Overbey, Katie

From: Mizue Naito <mizue@dosebiosystems.com>
Sent: Tuesday, February 15, 2022 2:46 PM
To: Overbey, Katie
Cc: Ted Jin
Subject: [EXTERNAL] Re: GRN 1022 - FDA's Follow-Up Comments

Hello Dr. Overbey,

Thank you very much for your questions regarding *S. salivarius* DB-B5. Please see below the responses to your questions:

Questions for GRN 1022

1. On p. 11, you state that finished food products containing *S. salivarius* DB-B5 will be labelled with appropriate allergen declarations (e.g., soy), as required under FALCPA. Please clarify if any components of the fermentation medium or other components of the manufacturing process are from an allergenic source.

Response:
The fermentation medium includes the use of soy peptone. This ingredient is affirmed as GRAS under 21 CFR §184.1553. Foods containing *S. salivarius* DB-B5 will be labelled with soy as an allergen, according to FALCPA requirements. No other components of the fermentation medium or other components of the manufacturing process are from an allergenic source.

2. Please clarify the following information about the microbial specifications provided in Table 2.3.1-1 on p. 12:
a. Please state the sample sizes used for the *Salmonella, Escherichia coli*, and bile tolerant gram-negative bacteria specifications and include these quantities in your specified limits for these microorganisms as well as in the results of batch analyses.

Response:
a. The sample sizes for *Salmonella* and *E. coli* are both 10 g, while sample size for bile tolerant Gram-negative bacteria are 5 g. Please see the updated relevant sections of tables below that include these quantities:

Table 2.3.1-1 Product Specifications for *S. salivarius* DB-B5 (Microbiology only)

Parameter	Specification	Method of Analysis
Microbiological Criteria		
Aerobic plate count (CFU/g)	NMT 50	USP<61>
Yeast and mold count (CFU/g)	NMT 50	USP<61>
Salmonella	Negative in 10 g	USP<62>
Escherichia coli	Negative in 10 g	USP<62>
Bile tolerant gram-negative bacteria	Negative in 5 g	USP<62>

CFU = colony forming units; NMT = not more than.

Table 2.3.2-1 Analytical Data from 3 Representative Lots of *S. salivarius* DB-B5 (Microbiology only)

Parameter	Specification	Lot Number		
		BR-PD-5	BR-PD-6	BR-PD-7
Microbiological Criteria				
Aerobic plate count (CFU/g)	NMT 50	<10	<10	<10
Yeast and mold count (CFU/g)	NMT 50	<10	<10	<10
Salmonella	Negative in 10 g	Negative in 10 g	Negative in 10 g	Negative in 10 g
Escherichia coli	Negative in 10 g	Negative in 10 g	Negative in 10 g	Negative in 10 g
Bile tolerant gram-negative bacteria	Negative in 5 g	Negative in 5 g	Negative in 5 g	Negative in 5 g

CFU = colony forming units; NMT = not more than.

b. Please confirm that the methods used for the aerobic plate count, yeast and mold count, and to enumerate *Salmonella, E. coli*, and bile tolerant gram-negative bacteria are each validated for the stated use and sample size.

Response:
b. The microbiology analysis was performed by an external laboratory testing company. The validation/suitability was performed by the company performing the analysis.

3. Please confirm that the internal method used to enumerate *S. salivarius* DB-B5 is validated for detection of the target microorganism at the listed sample size of 1g.

Response:
Our internal method for measuring CFU/g has been validated.

4. For the administrative record, please state if *S. salivairius* DB-B5 is non-pathogenic and non-toxigenic.

Response:
S. salivarius DB-B5 is non-pathogenic and non-toxigenic, according to our *in silico* analyses described in Section 6.5, and our clinical studies described in Section 6.4.

5. Regarding the intended uses (Table 3.1.2-1) on pp. 15-16:
a. Please clarify the intended use in milk (fresh) and cream (pasteurized) given the standards of identity for milk and cream. It is not clear that milk and cream with added *S. salivarius* DB-B5 would be distinct from the categories of cultured milk and cream, which you have also listed. Was this distinction intentional or would these intended uses be covered by the milk products category (21 CFR 170.3(n)(31))?

Response:
To clarify, Table 3.1.2-1 reflects the intended food uses that were listed in the GRAS notices for *S. salivarius* K12 (GRN 591) and *S. salivarius* M18 (GRN 807) submitted previously by BLIS Technologies Ltd. We are not sure why milk (fresh) and cream (pasteurized) are listed separately from "cultured milk products" by the notifier of GRN 591 and GRN 807. We agree that cultured milk and cream would be covered by the milk products category in 21 CFR 170.3(n)(31).

b. Please clarify the intended use in water (still or mineral). Does this refer to flavored waters that may or may not be carbonated? As written in the Table 3.1.2-1, this category appears to be bottled water and would be subject to the requirements under 21 CFR 165.110.

Response:
As explained in our response to Q.1a above, Table 3.1.2-1 reflects the intended food uses that were listed in GRN 591 and 807. We believe it is the intention of the notifier for those GRNs (BLIS Technologies Ltd.) that this category reflects flavored waters (that may or may not be carbonated), and not bottled water subject to the requirements of 21 CFR 165.110.

To clarify, Dose Biosystems would like to note that *S. salivarius* DB-B5 is intended for addition into standardized foods only if it is permitted by the applicable standard of identity.

6. Dietary exposure estimates should incorporate the maximum use levels of an ingredient and be based on current food consumption data. For the dietary exposure, please clarify or revise the following:
a. Was the use level assumed to be equivalent to the target level of 1×10^9 CFU/serving?

Response:
Yes, the target levels of *S. salivarius* DB-B5 in food would be 1×10^9 CFU/serving.

b. What is the maximum use level considered in your safety evaluation? If this level differs from the level used in your dietary exposure estimate, please provide dietary exposure estimates for the US population aged 2 years and older, infants/toddlers, and children based on the stated maximum use level. Please also confirm that you have concluded *S. salivarius* is GRAS for use at levels up to the proposed maximum use level.

Response:
The safety assessment of *S. salivarius* DB-B5 was based on the assumption that it will be present in foods at the target use level of 1x10^9 CFU/serving. Similar to other live microbial strains that have GRAS status as food ingredients, *S. salivarius* DB-B5 will be added to foods at an overage in order to account for loss of viability over time and ensure the target level (1x10^9 CFU/serving) is maintained throughout the shelf-life of the food product. The level of overage required will depend on the specific food application, though this typically ranges around 2- to 5-fold, and may reach as high as 10-fold. Thus, the initial addition level of *S. salivarius* DB-B5 into foods could potentially be as high as 1x10^10 CFU/serving.

With regards to the exposure calculation, the estimated daily intake values reported in Table 3.2-1 (pg. 17) were incorporated by reference from GRN 591 and GRN 807. In these previous GRAS notices, it appears the exposure calculation was derived assuming a use level of 1x10^9 CFU/serving, even though the intended use level was stated to provide a minimum of 1x10^9 CFU/serving. Nonetheless, to account for the possibility that the initial addition level of *S. salivarius* DB-B5 could potentially be 1x10^10 CFU/serving, the estimated daily intake is expected to be in the ranges of 10^11 CFU/day assuming that 20 servings of foods containing *S. salivarius* DB-B5 are consumed daily. This level of intake (10^11 CFU/day) is consistent with the ranges that have been estimated for various other viable lactic acid bacteria strains with GRAS status (e.g., GRN 840, GRN 856). It should also be reiterated that this is considered an extremely conservative estimate, as it assumes there is no loss in viability of the strain during shipping and storage, and that all foods an individual consumes daily will contain *S. salivarius* DB-B5.

Thus, Dose Biosystems has concluded that *S. salivarius* DB-B5 is GRAS for its intended uses as a general ingredient in conventional foods at a target level of 1x10^9 CFU/serving, while also taking into account that the initial

addition level into foods may be as high as 1x10^10 CFU/serving in order to ensure the target level will be maintained throughout the shelf-life of the food product.

Please let me know if there are any other questions that need addressing. Thank you very much for the opportunity to address these questions.

Sincerely,
Mizue Naito

On Wed, 2 Feb 2022 at 14:16, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

> Dear Dr. Naito,
>
> During our review of GRAS Notice No. 001022, we noted questions that need to be addressed. Please find the questions below.
>
> Please format your response such that each answer immediately follows the stated question. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice.
>
> We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.
>
> Thank you in advance for your attention to our comments.
>
> Best,
>
> Katie
>
> **Questions for GRN 1022**
>
> 1. On p. 11, you state that finished food products containing *S. salivarius* DB-B5 will be labelled with appropriate allergen declarations (e.g., soy), as required under FALCPA. Please clarify if any components of the fermentation medium or other components of the manufacturing process are from an allergenic source.
> 2. Please clarify the following information about the microbial specifications provided in Table 2.3.1-1 on p. 12:
>   a. Please state the sample sizes used for the *Salmonella*, *Escherichia coli*, and bile tolerant gram-negative bacteria specifications and include these quantities in your specified limits for these microorganisms as well as in the results of batch analyses.
>   b. Please confirm that the methods used for the aerobic plate count, yeast and mold count, and to enumerate *Salmonella*, *E. coli*, and bile tolerant gram-negative bacteria are each validated for the stated use and sample size.

3. Please confirm that the internal method used to enumerate *S. salivarius* DB-B5 is validated for detection of the target microorganism at the listed sample size of 1g.
4. For the administrative record, please state if *S. salivairius* DB-B5 is non-pathogenic and non-toxigenic.
5. Regarding the intended uses (Table 3.1.2-1) on pp. 15-16:

    a. Please clarify the intended use in milk (fresh) and cream (pasteurized) given the standards of identity for milk and cream. It is not clear that milk and cream with added *S. salivarius* DB-B5 would be distinct from the categories of cultured milk and cream, which you have also listed. Was this distinction intentional or would these intended uses be covered by the milk products category (21 CFR 170.3(n)(31))?
    b. Please clarify the intended use in water (still or mineral). Does this refer to flavored waters that may or may not be carbonated? As written in the Table 3.1.2-1, this category appears to be bottled water and would be subject to the requirements under 21 CFR 165.110.
6. Dietary exposure estimates should incorporate the maximum use levels of an ingredient and be based on current food consumption data. For the dietary exposure, please clarify or revise the following:

    a. Was the use level assumed to be equivalent to the target level of 1 x 10^9 CFU/serving?
    b. What is the maximum use level considered in your safety evaluation? If this level differs from the level used in your dietary exposure estimate, please provide dietary exposure estimates for the US population aged 2 years and older, infants/toddlers, and children based on the stated maximum use level. Please also confirm that you have concluded *S. salivarius* is GRAS for use at levels up to the proposed maximum use level.

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

Division of Food Ingredients

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Mizue Naito, Ph.D.
Director, Microbiome and Probiotics R&D
Dose Biosystems

MaRS Discovery District, 661 University Ave, Suite 1300 Toronto, ON, M5G 0B7, Canada

Responses from notifier to questions for GRN 1022

5/22/2022

1. We understand that the intended uses listed in Table 3.1.2-1 were taken from GRNs 591 and 807. However, the information provided in a GRAS notice is the responsibility of the notifier and conclusions pertaining to the general recognition of safety of the notified substance for its intended uses must be clearly stated in the notice. In regard to our previous question 5b in your 2/15/2022 amendment, please confirm that you, Dose Biosystems, intend to use this product in flavored waters that may or may not be carbonated and not bottled waters subject to the requirements of 21 CFR 165.110.

Response:
Dose Biosystems intends to use the product in flavoured waters which may or may not be carbonated. Bottled waters (subject to 21 CFR 165.110) is not an intended use of the product at this time.

2. Please provide the deposition number for S. salivarius DB-B5.

Response:
S. salivarius DB-B5 has been deposited at the International Depositary Authority of Canada (IDAC) under the accession number 160720-01.

3. In the 2/15/2022 amendment, you note that estimates for dietary exposure to *S. salivarius* DB-B5 are up to 10^{11} CFU/serving and that this is based on consumption of 20 servings of food per day containing *S. salivarius* DB-B5. However, in the original notice you indicated that the dietary exposure was estimated by presuming that half (10) of these servings of food would contain *S. salivarius* DB-B5 at levels up to 10^{10} CFU/serving. Please clarify if your dietary exposure estimate in the amendment was based on 20 servings or 10 servings of food containing S. salivarius DB-B5 at levels up to 10^{10} CFU/serving.

Response:
For clarity, the dietary exposure in the amendment was based on 20 servings of food containing maximum levels of 10^{10} CFU/serving. In our original notice, we had noted that 20 servings would be extremely unlikely, as this reflects the amount of ALL foods that would be typically consumed in a day (Basiotis et al., 200). Thus, the notice had indicated that 10 servings of food may be a more realistic approach in the derivation of exposure.

4. Please provide a statement that all materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

Response:
All materials used in the manufacturing process are food grade, and approved under the following regulations: 21 CFR §173, 21 CFR §168, 21 CFR §184 or 21 CFR §182. All other ingredients are GRAS for its intended uses in the U.S.

Overbey, Katie

From: Mizue Naito <mizue@dosebiosystems.com>
Sent: Wednesday, June 15, 2022 10:51 AM
To: Overbey, Katie
Cc: Ted Jin
Subject: Re: [EXTERNAL] Re: GRN 1022 - Additional Questions

Hello Dr. Overbey,

Please see the response below to your question:

Question: You note in your response to question 3 in your 5/22/22 amendment that your exposure estimate was based on 20 servings containing maximum levels of *S. salivarius* DB-B5 of 10^{10} CFU/serving. However, you state that you estimate an intake of 1 x 10^{11} CFU/person/day in your 2/15/22 amendment. Please clarify that the correct estimate for dietary intake is 2 x 10^{11} CFU/person/day.

Response: In our response on 2/15/22, we had indicated that the level of intake would be in the ranges of 10^11, but was not very clear on the exact amount. We would like to clarify that while our target dose of *S. salivarius* DB-B5 is 1 x 10^9 CFU/serving, an overage of up to 1 x 10^10 CFU/serving may be required to ensure target level throughout shelf-life of some food applications. Assuming 20 servings are consumed in a day, the estimate for dietary intake would be 2 x 10^11 CFU/person/day.

Thank you,
Mizue Naito

On Mon, 13 Jun 2022 at 14:10, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

> Hello Dr. Naito,
>
> We had an additional clarifying question for GRN 1022:
>
> - You note in your response to question 3 in your 5/22/22 amendment that your exposure estimate was based on 20 servings containing maximum levels of *S. salivarius* DB-B5 of 10^{10} CFU/serving. However, you state that you estimate an intake of 1 x 10^{11} CFU/person/day in your 2/15/22 amendment. Please clarify that the correct estimate for dietary intake is 2 x 10^{11} CFU/person/day.
>
> We request that you please reply to this email with your response within 10 business days. If you require more time, please reach out to me.

Thank you,

Katie

From: Overbey, Katie
Sent: Wednesday, May 25, 2022 2:06 PM
To: Mizue Naito <mizue@dosebiosystems.com>
Cc: Ted Jin <ted@dosebiosystems.com>
Subject: RE: [EXTERNAL] Re: GRN 1022 - Additional Questions

Hello Dr. Naito,

Thank you for your responses to our questions. I will follow-up if we require any additional information.

Katie

From: Mizue Naito <mizue@dosebiosystems.com>
Sent: Sunday, May 22, 2022 8:45 AM
To: Overbey, Katie <Katie.Overbey@fda.hhs.gov>
Cc: Ted Jin <ted@dosebiosystems.com>
Subject: [EXTERNAL] Re: GRN 1022 - Additional Questions

Hello Dr. Overbey,

Thank you for your questions regarding *S. salivarius* DB-B5. Please see below the responses to your questions:

Questions for GRN 1022

1. We understand that the intended uses listed in Table 3.1.2-1 were taken from GRNs 591 and 807. However, the information provided in a GRAS notice is the responsibility of the notifier and conclusions pertaining to the general recognition of safety of the notified substance for its intended uses must be clearly stated in the notice. In regard to our previous question 5b in your 2/15/2022 amendment, please confirm that you, Dose Biosystems, intend to use this

product in flavored waters that may or may not be carbonated and not bottled waters subject to the requirements of 21 CFR 165.110.

Response:

Dose Biosystems intends to use the product in flavoured waters which may or may not be carbonated. Bottled waters (subject to 21 CFR 165.110) is not an intended use of the product at this time.

2. Please provide the deposition number for S. salivarius DB-B5.

Response:

S. salivarius DB-B5 has been deposited at the International Depositary Authority of Canada (IDAC) under the accession number 160720-01.

3. In the 2/15/2022 amendment, you note that estimates for dietary exposure to *S. salivarius* DB-B5 are up to 10^{11} CFU/serving and that this is based on consumption of 20 servings of food per day containing *S. salivarius* DB-B5. However, in the original notice you indicated that the dietary exposure was estimated by presuming that half (10) of these servings of food would contain *S. salivarius* DB-B5 at levels up to 10^{10} CFU/serving. Please clarify if your dietary exposure estimate in the amendment was based on 20 servings or 10 servings of food containing S. salivarius DB-B5 at levels up to 10^{10} CFU/serving.

Response:

For clarity, the dietary exposure in the amendment was based on 20 servings of food containing maximum levels of 10^{10} CFU/serving. In our original notice, we had noted that 20 servings would be extremely unlikely, as this reflects the amount of ALL foods that would be typically consumed in a day (Basiotis et al., 200). Thus, the notice had indicated that 10 servings of food may be a more realistic approach in the derivation of exposure.

4. Please provide a statement that all materials used in the manufacturing process are approved for their respective uses via a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

Response:

All materials used in the manufacturing process are food grade, and approved under the following regulations: 21 CFR §173, 21 CFR §168, 21 CFR §184 or 21 CFR §182. All other ingredients are GRAS for its intended uses in the U.S.

Thank you very much,

Mizue Naito

On Thu, 19 May 2022 at 20:22, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Dear Dr. Naito,

During our review of GRAS Notice No. 001022, we noted additional questions that need to be addressed. Please find the questions below.

Please format your response such that each answer immediately follows the stated question. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice.

We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance for your attention to our comments.

Questions for GRN 1022

1. We understand that the intended uses listed in Table 3.1.2-1 were taken from GRNs 591 and 807. However, the information provided in a GRAS notice is the responsibility of the notifier and conclusions pertaining to the general recognition of safety of the notified substance for its intended uses must be clearly stated in the notice. In regard to our previous question 5b in your 2/15/2022 amendment, please confirm that you, Dose Biosystems, intend to use this product in flavored waters that may or may not be carbonated and not bottled waters subject to the requirements of 21 CFR 165.110.

2. Please provide the deposition number for *S. salivarius* DB-B5.

3. In the 2/15/2022 amendment, you note that estimates for dietary exposure to *S. salivarius* DB-B5 are up to 10^{11} CFU/serving and that this is based on consumption of 20 servings of food per day containing *S.* salivarius DB-B5. However, in the original notice you indicated that the dietary exposure was estimated by presuming that half (10) of these servings of food would contain *S. salivarius* DB-B5 at levels up to 10^{10} CFU/serving. Please clarify if your dietary exposure estimate in the amendment was based on 20 servings or 10 servings of food containing *S. salivarius* DB-B5 at levels up to 10^{10} CFU/serving.

4. Please provide a statement that all materials used in the manufacturing process are approved for their respective uses *via* a regulation in Part 21 of the U.S. Code of Federal Regulations, are the subject of an effective food contact notification, or are GRAS for that use in the U.S.

Best,

Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
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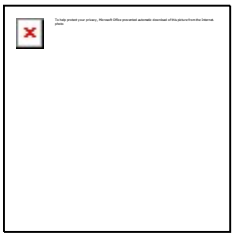

Mizue Naito, Ph.D.
Director, Microbiome and Probiotics R&D
Dose Biosystems

MaRS Discovery District, 661 University Ave, Suite 1300 Toronto, ON, M5G 0B7, Canada

Overbey, Katie

From: Mizue Naito <mizue@dosebiosystems.com>
Sent: Tuesday, August 2, 2022 10:33 PM
To: Overbey, Katie
Cc: Ted Jin
Subject: [EXTERNAL] Re: GRN 1022 - Follow-up Question

Hi Dr. Overbey,

Thank you very much for your question. Please see below our response:

Dose Biosystems notes that other closely related *S. salivarius* strains, namely *S. salivarius* K12 (GRN No. 591) and *S. salivarius* M18 (GRN No. 807), have been concluded GRAS for their intended uses across a broad range of food categories, including "baby, infant, and toddler foods (excluding infant formula)". Even though *S. salivarius* DB-B5 is manufactured under hygienic conditions in accordance with cGMP and HACCP to minimize the likelihood of microbial contamination, *Cronobacter sakazakii* is not currently included as a specification parameter. As such, Dose Biosystems would like to clarify that *S. salivarius* DB-B5 is intended only for use in conventional foods intended for the general population. ***S. salivarius* DB-B5 will not be used in infant formula, or other products that are intended for consumption by infants or very young children**. Additionally, *S. salivarius* DB-B5 will not be used in products where the standard of identity may preclude its use, or in meat and poultry products that are regulated by the FSIS of the USDA.

Thank you very much,
Mizue Naito

On Tue, 26 Jul 2022 at 11:25, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

> Hello Dr. Naito,
>
> We have noted an additional question for GRN 1022 that is provided below.
>
> We respectfully request a response within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.
>
> 1. *Cronobacter sakazakii* has been isolated from foods intended for very young children and can cause infection in infant and toddler populations. Because Dose Biosystems lists an intended use of *S. salivarius* DB-B5 as an ingredient in infant foods there remains a potential risk to these vulnerable populations if *C. sakazakii* is not controlled for during the production of *S. salivarius* DB-B5 or if foods formulated with this ingredient are not treated with an inactivation step (e.g., retort) before consumption by infants or toddlers. We note the following publications that discuss the prevalence and potential concerns of *C. sakazakii* presence in such foods:

- Chen, Q., Zhu, Y., Qin, Z., Qiu, Y., & Zhao, L. (2018). Cronobacter spp., foodborne pathogens threatening neonates and infants. *Frontiers of Agricultural Science and Engineering*, 5(3), 330-339.
- Forsythe, S. J. (2015). New insights into the emergent bacterial pathogen Cronobacter. In *Food Safety* (pp. 265-308). Academic Press.

Given that the intended uses include use in foods intended for consumption by infants and very young children, how does Dose Biosystems plan to control for the presence of *C. sakazakii*? If Dose Biosystems does not plan to include a specification for *C. sakazakii*, please provide a discussion regarding why this is not necessary from a safety perspective and how the presence of *C. sakazakii* is controlled during manufacture of *S. salivarius* DB-B5.

Thank you,

Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

Division of Food Ingredients

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Mizue Naito, Ph.D.
Director, Microbiome and Probiotics R&D
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