



## Soni & Associates Inc.

749 46<sup>th</sup> Square  
Vero Beach, FL 32968, USA  
Telephone: 772-299-0746  
Facsimile: 772-299-5381  
E-mail: [msoni@soniassociates.net](mailto:msoni@soniassociates.net)

May 21, 2019

#1864

Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety (HFS-200)  
5100 Campus Drive  
College Park, MD 20740

**Subject: GRAS Notification for the intended use of *Bacillus coagulans* SNZ 1969 spores preparation in Infant Formula**

Dear Sir/Madam:

In accordance with 21 CFR part 170, subpart E, Sanzyme Biologics Pvt. Ltd., through Soni & Associates Inc. as its agent, hereby submits the enclosed notice of a claim that the food ingredient *Bacillus coagulans* SNZ 1969 spores preparation for use in infant formula as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

As required, please find enclosed three copies of the notification. If you have any questions or require additional information, please feel free to contact me by phone at +1-772-299-0746 or by email at [sonim@bellsouth.net](mailto:sonim@bellsouth.net).

Sincerely,

Madhu G. Soni, PhD, FACN, FATS

Agent for  
Sanzyme Biologics Pvt. Ltd.  
INDIA

Enclosure: Three copies of GRAS notification



**GENERALLY RECOGNIZED AS SAFE (GRAS) EVALUATION  
OF *BACILLUS COAGULANS* SNZ 1969 FOR USES IN TERM  
INFANT FORMULA**

**Submitted by:**

Sanzyme Biologics Pvt. Limited  
Plot No. 13, Sagar Society  
Banjara Hills, Road Number 2  
Hyderabad – 500 034, Telengana  
INDIA

**Submitted to:**

U.S. Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
HFS-200  
5100 Campus Drive  
College Park, MD 20740  
USA

**Contact for Technical and Other Information**

Madhu G. Soni, PhD  
Soni & Associates Inc.  
749 46<sup>th</sup> Square  
Vero Beach, FL 32968

May 08, 2019

**GENERALLY RECOGNIZED AS SAFE (GRAS) EVALUATION OF  
*Bacillus coagulans* SNZ 1969  
 FOR USES IN TERM INFANT FORMULA**

**TABLE OF CONTENTS**

<b>1. Part 1 – SIGNED STATEMENTS AND CERTIFICATION .....</b>	<b>4</b>
1.1. Name and Address of Notifier .....	4
1.2. Name of Notified Substance .....	4
1.3. Intended Conditions of Use .....	4
1.4. Statutory Basis for GRAS Determination .....	5
1.5. Exclusion from Premarket Approval.....	5
1.6. Availability of Data & Information.....	5
1.7. Data Exemption from Disclosure .....	5
1.8. Certification.....	6
1.9. Name, Position/Title of Responsible Person who Signs the Dossier and Signature.	6
1.10. FSIS/USDA – Use in Meat and/or Poultry .....	6
<b>2. Part 2 – IDENTITY, SPECIFICATION AND MANUFACTURING .....</b>	<b>7</b>
2.1. Identity .....	7
2.1.1. Name and Source of GRAS Organism.....	7
2.1.2. Description of GRAS Organism .....	7
2.1.3. Identification and Characterization .....	8
2.1.3.1. Phenotypic Identification .....	8
2.1.3.2. Genotypic Identification.....	9
2.1.3.2.1. Genomic DNA isolation and quality assessment.....	9
2.1.3.2.2. rRNA gene PCR and phylogenetic analysis .....	9
2.1.3.2.3. Whole Genome Sequencing.....	11
2.1.4. Manufacturing Process.....	12
2.1.4.1. Seed Media Preparation .....	12
2.1.4.2. Seed Inoculation and Culture Growth.....	12
2.1.4.3. Sterilization, Fermentation and Separation .....	13
2.1.4.4. Drying, Sifting, Blending, Packaging .....	13

2.2.	Specifications .....	14
3.	Part III – DIETARY EXPOSURE .....	16
3.1.	Intended Use Levels and Food Categories.....	16
3.1.1.	Estimated Daily Intake from the Proposed Uses .....	16
4.	Part IV – SELF LIMITING LEVELS OF USE.....	18
5.	Part V – EXPERIENCE BASED ON COMMON USE IN FOODS BEFORE 1958 .....	19
6.	Part VI – NARRATIVE .....	20
6.1.	Data Pertaining to Safe Uses.....	20
6.1.1.	Traditional Uses .....	20
6.1.2.	Current Uses and Regulatory Status.....	20
6.2.	Safety Related Studies .....	23
6.2.1.	Studies in Infants and Children.....	23
6.2.2.	Human Studies with Labcon (Mother strain of SNZ 1969) .....	26
6.2.3.	Studies in Adult Human Subjects.....	27
6.2.4.	Safety Based on Genome Sequencing.....	35
6.2.5.	Specific Antibiotic Susceptibility Studies .....	36
6.2.6.	Specific Antimicrobial Action Studies .....	37
6.2.7.	Specific Bile Salt and Acid Tolerance Studies.....	38
6.2.8.	Animal Toxicity Studies .....	38
6.3.	Expert Panel Review, Summary and Discussion .....	44
6.4.	Expert Panel Conclusion .....	48
7.	Part VII – SUPPORTING DATA AND INFORMATION.....	49

## **1. Part I – SIGNED STATEMENTS AND CERTIFICATION**

### **1.1. Submission of GRAS Notice**

In accordance with 21 CFR § 170 Subpart E consisting of § 170.203 through § 170.285, Sanzyme Biologics Pvt. Ltd., (Sanzyme Biologics) hereby informs the FDA that *Bacillus coagulans* SNZ 1969 spores preparations manufactured by Sanzyme Biologics, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Sanzyme Biologics' view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below.

It should be noted that the subject of the present GRAS assessment, *B. coagulans* SNZ 1969 spore preparation, for use in term infant formula is identical to that of Sanzyme Ltd.<sup>1</sup> (the parent company Sanzyme Biologics) GRAS notice (GRN 597) that received a “no question letter” from FDA for the use of *Bacillus coagulans* SNZ 1969 spores preparation in conventional foods.

### **1.2. Name and Address of Notifier**

Sanzyme Biologics Pvt. Limited  
Plot No. 13, Sagar Society  
Banjara Hills, Road Number 2  
Hyderabad – 500 034, Telengana  
INDIA

### **1.3. Name of Notified Substance**

The common name of the substance of this GRAS assessment is *Bacillus coagulans* SNZ 1969 spores preparation for uses in term infant formula will be marketed as standardized powder.

### **1.4. Intended Conditions of Use**

*Bacillus coagulans* SNZ 1969 spore preparation (*B. coagulans*) is intended for use as an ingredient in non-exempt term infant formula at the maximum intended addition levels of  $2 \times 10^8$  colony forming units (cfu) per 100 mL infant formula as ready for consumption. *B. coagulans* is not intended for addition to pre-term formula. The intended uses and use levels of *B. coagulans* in term infant formula are identical to those described in GRN 660 (Ganeden, 2016).

---

<sup>1</sup> Sanzyme was having two divisions viz., Fermentation division dealing with manufacture and marketing of Probiotics and Probiotic formulations etc., and Healthcare division dealing with manufacture and / or marketing of pharmaceutical formulations, Nutraceuticals etc. In pursuance of approval of the scheme of demerger by National Company Law Tribunal India, The Ferments division of Sanzyme Pvt., Ltd., was demerged and vested into Sanzyme Biologics Pvt., Ltd., with effect from 1.4.2017. Sanzyme Biologics was demerged from Sanzyme Ltd in Jan 2018. Post demerger, Sanzyme Biologics is the company solely responsible for the production and sale of *B. coagulans* SNZ 1969

### 1.5. Statutory Basis for GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

### 1.6. Exclusion from Premarket Approval

Sanzyme Biologics has concluded that the use of *Bacillus coagulans* SNZ 1969 spores preparation is Generally Recognized As Safe, under the conditions of its intended use in non-exempt infant formula, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This GRAS conclusion has been reached in accordance with requirements in 21 CFR 170.220. Therefore, the use of *Bacillus coagulans* SNZ 1969 spores preparation is exempt from the premarket approval requirements of the FD&C Act.

### 1.7. Availability of Data & Information

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting Dr. Jaiswal or Dr. Soni at the below addresses. The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225I(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

Dr. Pallavi Jaiswal  
Manager- Technical Services & BD  
Sanzyme Biologics Pvt. Limited  
Plot No. 13, Sagar Society  
Banjara Hills, Road Number 2  
Hyderabad – 500 034, Telengana  
INDIA

Phone: +91-40-48589999  
E-mail: [probioticexports@sanzymebiologics.com](mailto:probioticexports@sanzymebiologics.com)

Or

Madhu G. Soni, PhD, FACN, FATS  
Soni & Associates Inc.,  
749 46<sup>th</sup> Square,  
Vero Beach FL, 32968

Phone: (772) 299-0746;  
E-mail: [sonim@bellsouth.net](mailto:sonim@bellsouth.net)

### 1.8. Data Exemption from Disclosure

Parts II through VII of this GRAS notification does not contain any data or information that is exempt from disclosure under the Freedom of Information Act. There is no privileged or confidential information such as trade secrets and/or commercial or financial information in this document and the information contained in this dossier can be made publicly available.

### 1.9. Certification

Sanzyme Biologics certifies that, to the best of its knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by Sanzyme Biologics, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of *Bacillus coagulans* SNZ 1969 spores preparation. Sanzyme Biologics accepts responsibility for the GRAS conclusion that has been made for *Bacillus coagulans* SNZ 1969 spores as described in this dossier.

### 1.10. Name, Position/Title of Responsible Person who Signs the Dossier and Signature

Dr. Pallavi Jaiswal  
Manager- Technical Services & BD  
Sanzyme Biologics Pvt. Limited  
Plot No. 13, Sagar Society  
Banjara Hills, Road Number 2  
Hyderabad – 500 034, Telengana  
INDIA

Phone: +91-40-48589999  
E-mail: [probioticexports@sanzymebiologics.com](mailto:probioticexports@sanzymebiologics.com)

Signature:  \_\_\_\_\_

### 1.11. FSIS/USDA – Use in Meat and/or Poultry

Sanzyme Biologics does not intend to add *Bacillus coagulans* SNZ 1969 spores to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

## 2. Part II – IDENTITY, SPECIFICATION AND MANUFACTURING

### 2.1. Identity

#### 2.1.1. Name and Source of GRAS Organism

The specific bacterial strain which is the subject of this Generally Recognized As Safe (GRAS) assessment is *Bacillus coagulans* SNZ 1969. It is a member of a subgroup of *Bacillus* spp. and is isolated as a spore-forming bacterium from green malt.

#### 2.1.2. Description of GRAS Organism

General descriptive characteristics and properties of the *B. coagulans* SNZ 1969 spore preparations manufactured by Sanzyme Biologics are summarized in Table 1. *B. coagulans* SNZ 1969 is a unique strain of spore forming *Bacillus* species. It is a gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. *B. coagulans* strain (SNZ 1969), the subject of the present GRAS determination, has been deposited with the Microbial Type Culture Collection (MTCC) - assigned number MTCC 5724 and with Belgian Coordinated Collections of Microorganism (BCCM<sup>TM</sup>/LGM) with the assigned number LMG S - 27484.

Additionally, the partial gene sequencing of this strain can be found at the National Center for Biotechnology Information database (Swamy and Soman, 2013) as well as at the DNA Data Bank of Japan (DDBJ). The spores of *B. coagulans* can withstand temperatures in excess of 100°C, while the vegetative cells can grow at temperatures as high as 65°C. *B. coagulans* is a highly resilient bacteria commonly found in the soil, air and dust. It can grow in a highly alkaline environment and the spores can also withstand the acidic environment of the stomach. The hierarchical classification of *B. coagulans* SNZ 1969 is presented in Table 2.

**Table 1. General Descriptive Characteristics of *Bacillus coagulans* SNZ 1969**

Parameter	Description *
Organism	<i>Bacillus coagulans</i> SNZ 1969
Origin	Isolated from green malt
Physical characteristics	A dark grayish white powder
Taste	Slightly sweet in taste
Odor	Characteristic
Shelf life	36 months

\*Based on information provided by Sanzyme Biologics

**Table 2. Classification of *Bacillus coagulans* SNZ 1969**

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Division	Endospore-Forming Bacteria
Phylum	Firmicutes
Class	Bacilli; Gram-Positive Endospore-Forming Bacteria
Order	Bacillales; Gram-Positive Endospore-Forming Rods
Family	Bacillaceae
Genus	<i>Bacillus</i>
Species	<i>Bacillus coagulans</i>
Strain	<i>Bacillus coagulans</i> SNZ 1969



## 2.1.3. Identification and Characterization

### 2.1.3.1. Phenotypic Identification

*Bacillus coagulans* is an annotated microorganism that has been well characterized. This strain originated in Japan. *B. coagulans* was first described in 1915 at the Iowa Agricultural Experiment Station associated with the coagulation of evaporated milk (Sarles and Hammer 1931). In 1949, a Japanese physician, Dr. Nakayama, isolated *B. coagulans* from green malt. This particular isolate was tested for its potential effects against diarrhea and constipation in adults as well as infants during 1964. In 1972, at the request of Sankyo Corporation, the Japanese Ministry of Health and Welfare approved the use of this particular *B. coagulans* (designated as strain SANK 70258). Subsequently, in 1973, Sankyo Corporation (currently known as Daiichi Sankyo Co. Ltd) offered formulation and fermentation technology to Sanzyme Biologics (earlier known as Uni-Sankyo Ltd). Since then, it is marketed in India under the brand name Sporlac<sup>2</sup> and has been designated as strain SNZ 1969. The phenotypic characteristics of *B. coagulans* SNZ 1969 are summarized in Table 3.

**Table 3. Phenotypic Characteristics of *Bacillus coagulans* strains SNZ 1969**

Test	<i>B. coagulans</i> SNZ 1969	Test	<i>B. coagulans</i> SNZ 1969
Gram staining	+	Dextrose	+
Catalase	+	Galactose	w
Gelatin hydrolysis	-	Raffinose	-
Nitrate reduction	-	Sucrose	-
Oxidase	+	L-Arabinose	-
Lactose	-	Inulin	w
Xylose	-	Sorbitol	-
Maltose	+	Mannitol	-
Fructose	+	Rhamnose	w

\*Based on information provided by Sanzyme Biologics; += positive; - = 90% negative, w = weak positive reaction

Losada and Olleros (2002) compared the differential characteristics between *Lactobacillus* and *Bacillus* species, including *B. coagulans*. This comparison is summarized in Table 4. These investigators suggested that the capacity of *B. coagulans* to form spores is a differential characteristic compared to other strains of *Lactobacillus*. The spore formation is a microencapsulation process in which a covering of calcium-dipicolinic acid-peptidoglycan complex is generated. This allows a high degree of stability in unfavorable conditions such as changes in humidity and temperature during storage or alterations in the gastrointestinal tract.

<sup>2</sup> It should be noted that *B. coagulans* SNZ 1969 is marketed in India under the name Sporlac. However, in the USA Sabinsa Corporation, who markets their Probiotic product *Bacillus coagulans* in the brand name 'LACTOSPORE' holds the trademark 'Sporlac'.

**Table 4. Differential Characteristics of *Lactobacillus* and *Bacillus* species**

Characteristics	<i>Bacillus</i> sp.	<i>Bacillus coagulans</i>	<i>Lactobacillus</i> sp.	Sporolacto bacillus
Catalase	+	+	-	-
Benzidine	+	NA	-	-
Nitrate red	+	NA	-	-
Gram-reaction	+	+	+	+
Endospores	+	+	-	+
Motility	+	+	- <sup>1</sup>	+
Lactic acid	- <sup>2</sup>	+	+	+
m-A2-PM <sup>3</sup>	+	+	- <sup>1</sup>	+
Fatty acid	Bacillus type	Lactobacillus type	Bacillus type	

<sup>1</sup>Except *L. plantarum*; <sup>2</sup>Apart from *B. coagulans* other species can produce lactic acid; <sup>3</sup>Meso-diaminopimelic acid; NA = No data available; Adapted from Losada and Olleros (2002)

### 2.1.3.2. Genotypic Identification

In an attempt to genetically characterize *B. coagulans* SNZ 1969, genotypic identification was carried out. The NCBI accession number for the 16S rRNA gene sequence of *B. coagulans* is KC146407. The phylogenetic characterization based on 16S rRNA and as compared to other related species and designates was studied (Swamy and Soman, 2013). To assign strains to bacterial species for each isolate almost the entire 16S rRNA gene was amplified. The 1491 bp amplicon was then sequenced using Sanger sequencing method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. The partial sequence is presented in Appendix I. In order to fully characterize *Bacillus coagulans* SNZ 1969, whole genome sequencing (WGS) was carried out.

#### 2.1.3.2.1. Genomic DNA isolation and quality assessment

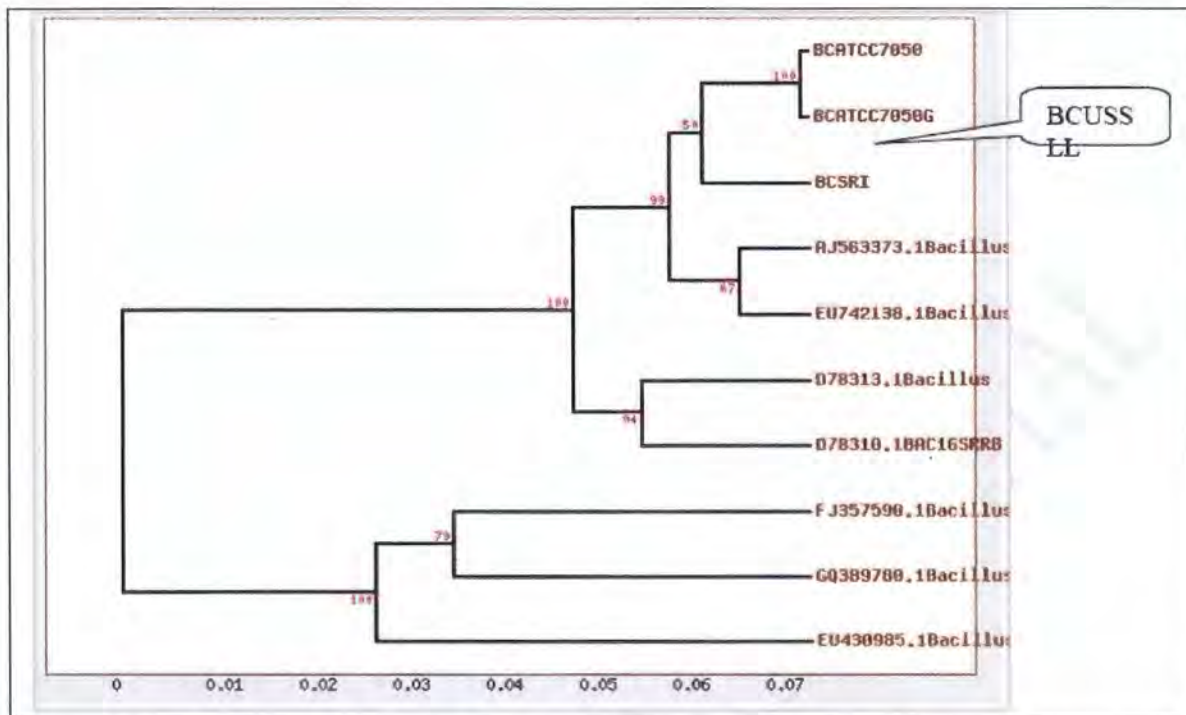
For genomic identification, *B. coagulans* strains, SNZ 1969 (referred for this analysis as BCUSS) and ATCC 7050<sup>3</sup> cultures were grown on Sterile PNY medium (HiMedia) for 24 hours at 37°C in a temperature controlled incubator. The cells were then resuspended in 0.9% saline and pelleted for further processing and isolation of genomic DNA. For nucleic acid isolation, STE buffer (0.1 M Tris-HCl, 0.1 M NaCl and 1 mM EDTA pH 8.5), Lysozyme and Proteinase K (10 µg/ml) were used. This method employed NaCl and SDS lysis followed by phenol: chloroform: iso-amyl alcohol purification of nucleic acids. The DNA was then deproteinised thrice with Tris-saturated phenol (Phenol: CHCl<sub>3</sub>: iso-amyl alcohol, 50:48:2), and then with CHCl<sub>3</sub>: isoamyl alcohol (24:1). DNA was then precipitated with 2% sodium acetate and absolute ethanol. Dried DNA was dissolved in nuclease free water. Quality assessment of genomic DNA was performed by 1% agarose gel electrophoresis as well as DNA was quantified using Qubit<sup>TM</sup> Fluorometer (Invitrogen, USA) for measurement of DNA concentration. Genomic DNA isolated from the cultures was high quality and was used for additional studies.

#### 2.1.3.2.2. rRNA gene PCR and phylogenetic analysis

The phylogenetic characterization based on 16S rRNA, and as compared to other related species and designates, was studied. Polymerase chain reaction based amplification of the 16S

<sup>3</sup> *B. coagulans* strain deposited with the American Type Culture Collection (ATCC) facility as *B. coagulans* Hammer, by Dr. N.R. Smith, and was given the designation number 7050.

rRNA gene was carried out using a Microbial Identification kit. The kit is comprised of two different primer sets targeting 16S rDNA from bacteria. Two overlapping fragments are generated that spans across more than 1300 bases of the 16S rRNA gene of bacteria. Amplification was carried out in a Gene Amp PCR System (Applied Biosystems, USA). The amplified DNA fragments of approximately 780 bp and 890 bp separated on a 2% agarose gel and purified by using PCR purification Kit. Sequencing products were precipitated, cleaned and loaded onto an automatic DNA Sequencer (ABI Prism Model 3130, Applied Biosystems, California, USA) for sequence analysis using Sequencing Analysis software version 5.2. The amplification for both the samples was confirmed by agarose gel electrophoresis on 2% gel. The PCR products were purified using PCR purification kit to remove unused dNTPs and primers. The purified PCR products were again checked by gel electrophoresis and then used for DNA sequencing. The DNA sequence was trimmed and edited during quality assessment and then used for phylogenetic analysis. The finding from this analysis is presented in Figure 1. The findings from this analysis indicate that *B. coagulans* SNZ 1969 (BCUSS) is closely related to *B. coagulans* ATCC 7050.



**Figure 1. The phylogenetic tree of *B. coagulans* SNZ 1969 (BCUSS) with the different *Bacillus* reference sequences.**

Distance-based phylogenetic methods are widely used in studies on molecular phylogenetics and evolution. The popularity of the distance based methods arises not only from their speed and performance which allows them to build super-trees, but also from their applicability to non-sequence data.

Distance-matrix methods of phylogenetic analysis explicitly rely on a measure of "genetic distance" between the sequences being classified, and therefore they require an MSA (multiple sequence alignment) as an input. It measures the pair-wise distance/dissimilarity between two genes, the actual size of which depends on different definitions, and constructs the tree totally from the resultant distance matrix.

The numbers at the node represent the percent bootstrap support for 1000 replicates. Bars at the base of the tree show genetic divergence. Description of Phylogeny: BCATCC7050: *Bacillus coagulans* ATCC 7050 strain reference sequence; BCATCC7050G: *Bacillus coagulans* strain ATCC 7050 sequence generated at our lab; BCUSS: *Bacillus coagulans* USS sequence generated at our lab; AJ563373.1: *Bacillus coagulans* reference sequence; D78313.1: *Bacillus coagulans* strain; EU742138.1: *Bacillus coagulans* strain SKU 12 reference sequence; D78310.1: BAC16SRRB *Bacillus badius* strain ATCC14574 reference sequence; FJ357590.1: *Bacillus ginsengihumi* strain BBNIR2-01 reference sequence, GQ389780.1 *Bacillus acidicola* strain TSAS-1 reference sequence; EU430985.1: *Bacillus oleronius* isolate 2 reference sequence. Note: All reference sequences used from NCBI database. Distance Matrix for these strains is presented below:

	1	2	3	4	5	6	7	8	9	10
1 BCATCC7050	0.000	0.001	0.011	0.009	0.022	0.021	0.017	0.058	0.063	0.065
2 BCATCC7050G	0.001	0.000	0.012	0.010	0.023	0.022	0.018	0.059	0.064	0.066
3 BCUSS	0.011	0.012	0.000	0.013	0.026	0.026	0.021	0.064	0.071	0.073
4 AJ563373.1	0.009	0.010	0.013	0.000	0.024	0.023	0.008	0.061	0.069	0.071
5 D78313.1	0.022	0.023	0.026	0.024	0.000	0.018	0.032	0.074	0.081	0.083
6 D78310.1	0.021	0.022	0.026	0.023	0.018	0.000	0.031	0.074	0.080	0.083
7 EU742138.1	0.017	0.018	0.021	0.008	0.032	0.031	0.000	0.067	0.075	0.077
8 FJ357590.1	0.058	0.059	0.064	0.061	0.074	0.074	0.067	0.000	0.050	0.038
9 EU430985.1	0.063	0.064	0.071	0.069	0.081	0.080	0.075	0.050	0.000	0.044
10 GQ389780.1	0.065	0.066	0.073	0.071	0.083	0.083	0.077	0.038	0.044	0.000

### 2.1.3.2.3. Whole Genome Sequencing

In an unpublished report (Heikkinen, 2017), whole genome sequencing (WGS), identification and bioinformatics safety assessment of *Bacillus coagulans* SNZ 1969 was carried out. *B. coagulans* de novo whole genome sequencing was done by GATC Biotech AG. A standard genomic PacBio library was produced from *B. coagulans* DNA, and sequencing was performed using PacBio RS technology. Several attempts were made to extract plasmid DNA, but it was not obtained, leading to the conclusion that plasmids are not present in this strain. The sequencing produced 90,720 raw reads and 1,003,270,000 sequenced bases. After filtering the number of reads was 76,973, the number of bases 872,855,228, N50 read length 16,590 bp, mean read length 11,339 bp and mean read score 0.84. The genome was sequenced and de novo assembled into three contigs with total length of 3,676,975 bp. The contigs were evaluated to be a good representation of the genome without significant gaps. The assembled *B. coagulans* genome sequences show high similarity with the reference strain (36D1), indicating that the contigs form a good representation of the genome. The genome was identified unequivocally as *B. coagulans*. As described later, genes known to confer resistance to antibiotics relevant to human or veterinary importance were not found in the genomic sequences. Neither were toxin genes or hazardous virulence factors found in the genome.

In an independently published study, Khatri et al. (2016) assembled *de novo* complete circular genomes of *B. coagulans* SNZ 1969 using HGAP v2.0, from PacBio reads obtained using the P6C4 chemistry. These investigators obtained a single contig of 3.69 Mbp

corresponding to a complete and circular chromosome for *B. coagulans* SNZ 1969. For this strain, the replication origin was identified at 1,091,681-1,092,581 bp and the corresponding *dnaA* gene was located downstream of the replication origin at 1092593–1093942 bp. It showed maximum similarity with *B. coagulans* 36D1 replication origin (ORI95010925, 912 bp) with an E-value of 0.0, 99% identity, and 100% coverage.

#### **2.1.4. Manufacturing Process**

The manufacturing process used in the production of *B. coagulans* SNZ 1969 for use in infant formula is identical to that of Sanzyme Biologics GRAS notice (GRN 597) on *Bacillus coagulans* SNZ 1969 spores preparation that received no question letter from FDA for the use of *Bacillus coagulans* SNZ 1969 spores preparation in conventional foods.

*B. coagulans* SNZ 1969 spore preparation is manufactured according to current good manufacturing practices (GMP) at Sanzyme Biologics Pvt. Limited, Plot Nos. 19 to 22, Sy Nos. 321/1,321/11,321/12,321/13,276 & 277, Karakapatla (Village), Markook (Mandal), Siddipet (District) Pin Code : 502 281, India. The FDA registration number for the manufacturing facility is 06/MD/AP/2013/B/G. The manufacturing process is schematically presented in Figure 2. The manufacturing procedure assures a consistent and high-quality product that meets the specifications (Table 5). The processing aids, fermentation medium and diluents used in the manufacturing of *B. coagulans* SNZ 1969 are either approved as food additives or are GRAS substances. The manufacturing facility is ISO 9001:2008 and ISO 22000:2005 certified. Standard Operating Procedures (SOPs) are followed and verification methods are in place. The manufacturing of *B. coagulans* SNZ 1969 spores involves the following steps:

##### **2.1.4.1. Seed Media Preparation**

The media ingredients, such as peptone as a nitrogen source, dextrose as carbohydrate source, and Corn Steep Liquor as vitamins and trace elements source, were used to support growth of the strain. All ingredients used are food grade and appropriate for such use. For the media preparations, accurately weighed media ingredients, are transferred carefully into a vessel that contains water. The ingredients are dissolved thoroughly and the final volume is adjusted to the required quantity. The pH of the medium is adjusted to 6.0 using food grade hydrochloric acid or sodium hydroxide. After adjusting the final pH of the media, the media is transferred to flasks and plugged with cotton, covered with butter paper and tied with thread, and loaded in to an Autoclave. The autoclave temperature is raised to  $122\pm 1^\circ\text{C}$  at  $1.2\pm 0.1 \text{ Kg/cm}^2$  pressure and maintained for one hour.

##### **2.1.4.2. Seed Inoculation and Culture Growth**

The sterilized seed medium flasks are brought to an Aseptic room, kept in Laminar Air Flow and the covered butter paper is removed. A loop full of culture from stock culture is inoculated into the seed flask. All this process is conducted under aseptic conditions. After completion of inoculation, the seed flask is covered with the cotton plug and wrapped with butter paper and tied with thread and labeled. The seed flasks are kept on an Orbital Shaker in the shaker room with constant shaking. Shaker room temperature is maintained at  $37\pm 1^\circ\text{C}$  using hot air generators. Seed flasks are kept (shaking) for minimum 16 - 20 hours and the growth of seed culture is checked under Phase contrast microscope.

#### **2.1.4.3. Sterilization, Fermentation and Separation**

The sterilization of the media in the fermenter is done at temperature 121°C for 45-60 min. Steam is used to attain this temperature. After the fermenter media reaches the desired temperature it is maintained for 60 minutes. Following this, the fermenter temperature is allowed to cool to 37±1°C by circulating chilled water generated by a Chiller. Following completion of sterilization, the steam inlet valve is closed. The seed culture that was grown in the seed flasks on shakers was inoculated. After complete transfer of inoculums into the fermenter, the inoculation tank bottom valve is closed. The steam is passed through the inoculation tank for 20 minutes. The pressure is maintained at 1.2±0.1 Kg/cm<sup>2</sup> and temperature 37±1°C. The air flow is maintained at 350-400 LPM, agitation kept at 125-150 RPM and agitation RPM is set based on pH and growth pattern. Every hour the Batch Manufacturing Record reading is noted. The organism growth is observed under microscope every hour. Once the sporulation is noted, pH is maintained with no further change. Chilled water is circulated through the jacket and the temperature is brought down below 28°C. The matured spore media is added to the separator and centrifuged at 7500 rpm.

#### **2.1.4.4. Drying, Sifting, Blending, Packaging**

The separated wet biomass is transferred to a spray dryer receiver tank and dried under standardized conditions. The powder thus obtained is mixed and passed through #100 mesh by using a sifter. The trays are removed and the material is spread for drying and placed in Tray dryer and subjected to desired temperature. After completion of drying, the material is allowed to cool and unloaded into a blender. Before unloading the blend a sample is collected for analysis. The powder is collected into double-layered polythene bags that are kept in labeled HPDE drums. The blending and collecting process is repeated to obtain the desired material. In order to obtain the desired final concentration of spores, the powder is mixed with food grade maltodextrin or lactose.

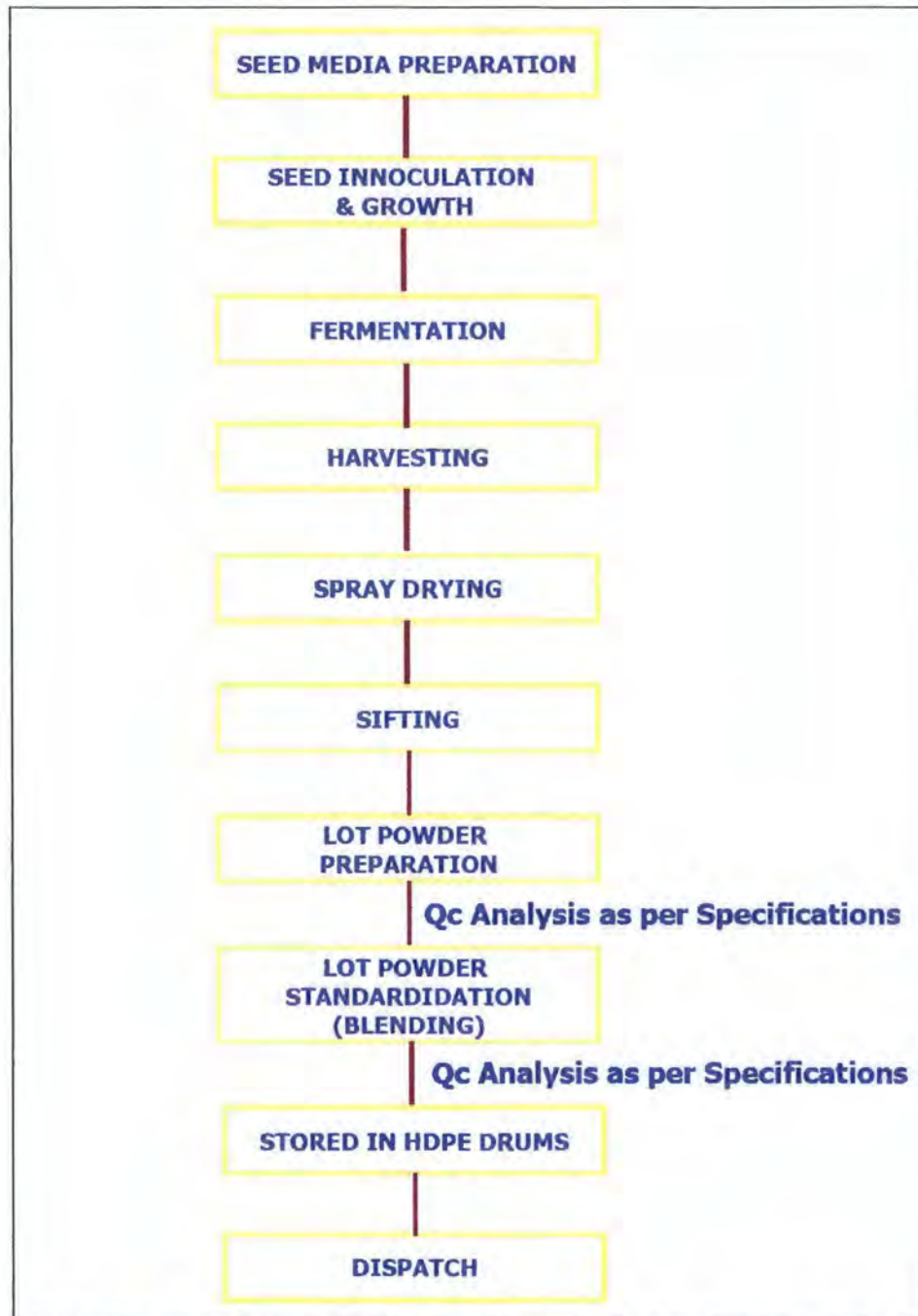


Figure 2. Manufacturing process of *Bacillus coagulans* SNZ 1969 (Sanzyme Biologics, 2018)

## 2.2. Specifications

Food grade specifications of *B. coagulans* SNZ 1969 spore preparations have been established by Sanzyme Biologics and are presented in Table 5. Analytical results from five non-consecutive lots (Appendix II) demonstrate that *B. coagulans* SNZ 1969 is consistently manufactured to meet these specifications. The *B. coagulans* SNZ 1969 strain, the subject of the present GRAS assessment was found, through 16s rDNA Analysis, to be over 99% similar to *B.*

*coagulans* type strain ATCC 7050. The major allergens like gluten, nuts, milk and dairy products are not present in the final spore preparation.

**Table 5. Specifications of *Bacillus coagulans* SNZ 1969 preparation**

Parameter	Characteristics (Sanzyme Biologics, 2018)*
Appearance	Dark grayish white powder
Identification	Aerobic, gram positive thermostable spores
Loss on drying (105° for 1 hour)	NMT 6% w/w
Viable spore	NLT 5x10 <sup>10</sup> spores/g
Lactic acid producing capacity	NLT 10 ml of 0.05 N NaOH consumed
<b>Heavy metals</b>	
Arsenic	NMT 1 ppm
Lead	NMT 1 ppm
Mercury	NMT 1 ppm
Cadmium	NMT 1 ppm
<b>Microbiological assays</b>	
Total bacterial counts (other organisms)	NMT 0.1 million cfu/g
Yeast and Mold	NMT 10 cfu/g
<i>Escherichia coli</i>	Negative/10 g
<i>Salmonella</i>	Negative/10 g
<i>Pseudomonas aeruginosa</i>	Negative/1 g
<i>Staphylococcus aureus</i>	Negative/1 g
<i>Bacillus cereus</i>	Negative/1 g
<i>Lysteria monocytogens</i>	Negative/25 g
<i>Cronobacter sakazakii</i>	Negative/100g

\*Based on information provided by Sanzyme Biologics; NMT = Not more than; NLT = Not less than; cfu = colony forming units; ppm = parts per million.



### 3. Part III – DIETARY EXPOSURE

#### 3.1. Intended Use Levels and Food Categories

Sanzyme Biologics intends to use a *B. coagulans* SNZ 1969 spore preparation as a food ingredient in non-exempt term infant formula (including powder, liquid concentrates, and ready-to-feed formulas) at a maximum addition levels up to  $2 \times 10^8$  cfu per 100 ml infant formula as ready for consumption (from birth to approximately 6 months).

##### 3.1.1. Estimated Daily Intake from the Proposed Uses

The proposed uses and use levels of *B. coagulans* SNZ 1969 spore preparation are the same as those described in a previous GRAS notice GRN 660 (Ganeden, 2016) for use of *B. coagulans* GBI-30, 6086 spores. In the GRAS notice by Ganeden, the resulting exposures to *B. coagulans* from the proposed uses has been estimated. *B. coagulans* SNZ 1969 spore preparation by Sanzyme Biologics for use in the term infant formula is at identical use levels, mentioned in the GRN 660. There are no new food uses proposed by Sanzyme Biologics for *B. coagulans* SNZ 1969 spore preparation. The intended use of *B. coagulans* SNZ 1969 spore preparation in the same foods and at the same levels as those in GRN 660 is not expected to noticeably affect the intake of *B. coagulans* SNZ 1969 spore preparation in the formula fed infants from introduction into the market by another supplier who will have to compete in essentially the same markets and foods.

In the estimates reported by Ganeden (2016) in GRN 660, daily energy intake by formula fed children was considered in estimating *B. coagulans* intake. For these assessments, daily energy intake of infants fed infant formula provided by Fomon (1974) were considered. As Fomon (1974) data is old, in GRN 660, more recent information from the 2005-2012 National Health and Nutrition Examination Survey (NHANES) based on the What We Eat In America (WWEIA) food category classification system (Grimes et al., 2015) and the Feeding Infants and Toddlers Study (FITS) 2008 (Butte et al., 2010) were considered. The previous report by Fomon was found to be somewhat consistent with recent information. From these publications, the most conservative estimates of 143 kcal/kg bw/day (the 90th percentile in girls 8-13 days and boys 14-27 days) and 67 kcal/100 mL formula as ready to consume from the majority of standard formulas and assuming formula accounts for 100% of energy consumption, approximately 213.4 mL/kg bw/day of infant formula would be consumed. Additionally, in GRN 660, it was noted that the majority of standard ready to consume formulas contain 67 kcal/100 ml.

Considering the above information in GRN 660, the intake of *B. coagulans* was calculated. The maximum use levels of  $2 \times 10^8$  cfu *B. coagulans*/100 ml of infant formula as ready for consumption was determined to result in a conservative high-end estimated daily intake of  $4.27 \times 10^8$  cfu *B. coagulans*/kg bw. As the proposed use levels and the food category (term infant formula) is the same, the proposed use by Sanzyme Biologics of *B. coagulans* SNZ 1969 spore preparation will also result in maximum intake of  $4.27 \times 10^8$  cfu *B. coagulans*/kg bw. As *B. coagulans* is GRAS for use in a variety of foods as described in GRN 597 and other GRAS notices, it is likely that introduction of other conventional foods to infants during weaning from the formula, exposure to *B. coagulans* may occur from both infant formula as well as conventional food. Introduction of any foods that might possibly contain *B. coagulans* would be at the expense of formula (i.e., to maintain the same caloric intake, formula consumption would necessarily decrease as solid foods are added) and it is quite likely that the result of introduction

of other foods would be a net decrease to *B. coagulans*. Any potential exposure from conventional foods is expected to be largely substitutional to exposure from infant formula and is likely to remain well within an acceptable margin of safety.

#### **4. Part IV – SELF LIMITING LEVELS OF USE**

No known self-limiting levels of use are associated with the notified ingredient *B. coagulans* SNZ 1969 spore preparation. As such, users will control the amounts used due to economic reasons.

## **5. Part V – EXPERIENCE BASED ON COMMON USE IN FOODS BEFORE 1958**

Not applicable. The statutory basis for the conclusion of GRAS status of *B. coagulans* SNZ 1969 spore preparation in this document is not based on common use in food before 1958. The GRAS conclusion for *B. coagulans* SNZ 1969 spore preparation is based on scientific procedures

## 6. Part VI – NARRATIVE

### 6.1. Data Pertaining to Safe Uses

#### 6.1.1. Traditional Uses

The available information suggest that lactic acid producing bacteria have been used in foods for centuries and these microorganisms are generally considered as harmless (Lee and Salminen, 1995). These bacteria are commonly used as starter cultures for fermentation in the dairy, meat and other food industries. Several strains selected for such uses have been previously associated or are endogenously found in humans. Such a selection ensures the safety of these bacteria in food. The inherent properties of these microorganisms have been utilized in the manufacturing of products such as cheese, yoghurts, fermented milk products, beverages, sausages, and olives. The available evidence also indicates that these bacteria can also improve the safety, shelf life, nutritional value, flavor and quality of the product. As discussed below, lactic acid bacteria can be used as cell factories for the production of food additives or enzyme preparations. These bacteria may also function as probiotics and contribute to the well-being of humans.

Traditionally, several lactic acid producing bacteria have been consumed in the diet. For the past several decades, the role of lactic acid bacteria has been extensively studied in the intestinal microecology. These bacteria play an important role in maintaining the healthy digestive tract (Catanzaro and Green, 1997; Adams, 1999; Soomro et al., 2002; Ouwehand et al., 2004). *B. coagulans* was first isolated in 1932 (Horowitz-Wlassowa and Nowotelnow, 1932) and has been used in the production of food products. In a series of studies from Portugal during 1958 and 1959, the potential gastrointestinal benefits of *B. coagulans* and other spore-forming bacteria have been investigated, including studies in children age 3 to 5 years (Guida et al., 1958; Guida and Guida, 1959). In a review article, Sanders et al. (2003) reported that among the 77 recognized *Bacillus* species, five species, including *B. coagulans*, have been evaluated for probiotic functionality and sold worldwide for both human and animal uses. The available information suggest that *B. coagulans* has been in use for over 50 years.

In Africa, dietary consumption of fermented foods has a long history (Okonko et al., 2006). In the Ibo ethnic group of Nigeria, *ugba* is a popular protein-rich solid, flavorful alkaline food, among other fermented foods. *B. coagulans* is one of the species identified in the preparation of *ugba*. It is produced by fermentation of African oil bean with *B. coagulans*. Consumption of *ugba* is known to result in the intake of *B. coagulans* vegetative form and its spores (Isu and Njoku, 1997). The available information indicate that a large proportion of the population (76%) has been reported to consume *ugba* as a snack (Onofiok et al., 1996). The presence of bacillus cells in *ugba* supports the intake of *B. coagulans*. The level of bacteria (*B. coagulans*) present in the *ugba* indicates that consumption of *B. coagulans* is greater than  $1 \times 10^9$  cfu/day. This provides support for the traditional use and consumption of *B. coagulans*.

#### 6.1.2. Current Uses and Regulatory Status

The available information shows that spore-forming bacteria, such as *B. coagulans* and *B. subtilis*, are used as dietary supplement probiotics for human consumption (Sanders et al., 2003; Hong et al., 2008). As a dietary supplement, *B. coagulans* is marketed as probiotics for human consumption to improve and maintain ecological balance of the intestinal microflora. At present, across the world, *B. coagulans* has been sold as a dietary supplement under different names such

as Ganeden BC30, Nature's Plus, Sunwarrior probiotics (SNZ 1969), Super Flora (SNZ 1969) GutFlor, Sporlac® (SNZ 1969), Sanvita, Ampilac, Bactolyte, Ba-Co-Flor, etc. Additionally, it is also marketed as a constituent with several other products. These formulations contain *B. coagulans* alone or in combination with lactobacilli or bifidobacteria, minerals, vitamins (particularly B complex), and prebiotics. The recommended dose of *B. coagulans* ranges from  $3.6 \times 10^8$  -  $1.5 \times 10^9$  cfu/capsule, two or three times *per day* for a healthy adult. Catanzaro and Green (1997) suggested a standard dose of *B. coagulans* at levels of  $1.5 \times 10^9$  cfu once or twice per day.

In the US, as dietary supplements<sup>4</sup>, *Bacillus coagulans* has been in use before 1994<sup>5</sup>. As a supplement, *B. coagulans* and its preparations are marketed under the Dietary Supplement Health and Education Act (DSHEA, 1994). The available information from the National Institute of Health reveals: 4 products which contain "*Bacillus Coagulans*" in the product name; 5 ingredient name(s) which contain "Bacillus Coagulans"; and 309 products which contain "Bacillus Coagulans" anywhere on the label (ODS/NLM, 2019). In a recent review article on the potential use of *B. coagulans* in the food industry, Konuray and Erginkaya (2018) reported that many food products such as Nutrition essentials Probiotic; NutriCommit; Flora3; THORNE; Sunny Green Cleansing; Just Thrive; MegSporeBiotic; Sustenex; and Neolactoflorene containing *B. coagulans* have been sold in various countries.

In Europe, the European Food Safety Authority granted a Qualified Presumption of Safety (QPS) status for *B. coagulans* since 2008 (EFSA, 2012). In Japan, the Japanese Ministry of Health and Welfare has approved the use of *B. coagulans* product (Lacbon) for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998). In Japan, Sankyo Corporation marketed the *B. coagulans* (SANK 70258) product under the trade name Lacbon. In India, *B. coagulans* is approved and has been marketed for the past four decades under the brand name Sporlac<sup>6</sup> by Sanzyme. In 1969, Uni Sankyo Ltd (now known as Sanzyme Pvt., Ltd) was incorporated as an Indo Japanese joint venture in collaboration with Sankyo Co. In 1973, Uni-Sankyo Ltd received the *B. coagulans* (SANK 70258) strain from Sankyo along with its manufacturing technology. In the past eight years (2011-2018), Sanzyme or Sanzyme Biologics has manufactured more than 6,000 tonnes of *B. coagulans* SNZ 1969 ( $5 \times 10^9$  spores/g) and has marketed it in various countries, including India, European countries, USA, Korea, Indonesia, etc., without reports of any significant adverse reports.

In the USA, FDA (2001) has approved the use of *B. coagulans* in the production of enzymes that are used for food production. As per 21 CFR 184.1372, *B. coagulans* (a nonpathogenic and nontoxicogenic microorganism) is recognized as GRAS in the production of insoluble glucose isomerase enzyme. Additionally, FDA's Center for Veterinary Medicine has approved the use of *B. coagulans* as GRAS for veterinary purposes. Similarly, Health Canada has permitted the use of *B. coagulans* in the production of glucose isomerase enzyme.

---

<sup>4</sup> See: <https://dslid.nlm.nih.gov/dslid/rptQSearch.jsp?item=Bacillus+Coagulans&db=adslid>

<sup>5</sup> NNFA List of Dietary supplements in Use Before October 15, 1994; available at: <http://www.fda.gov/ohrms/dockets/dockets/05p0305/05p-0305-cr00001-03-NNFA-List-voll.pdf>.

<sup>6</sup> As mentioned earlier, *B. coagulans* SNZ 1969 is marketed in India under the name Sporlac. In the US, Sabinsa Corporation who markets their Probiotic product *Bacillus coagulans* in the brand name 'LACTOSPORE' holds the trademark 'Sporlac'.

In addition to the use of *B. coagulans* in the production of enzymes, FDA has evaluated several GRAS notices on the use of *B. coagulans* in food. As of now, FDA has received eight GRAS notices on *Bacillus coagulans* for its use in food products. In August 2011, the FDA received the first GRAS notice (GRN 399), on the use of *B. coagulans* spore preparation in conventional foods, submitted by Ganeden Biotech Inc. (Ganeden, 2011). The intended maximum use level of *B. coagulans* preparation was  $2 \times 10^9$  cfu/serving in multiple food categories. The estimated daily intake of *B. coagulans* spores from all uses was determined as  $36.4 \times 10^9$  cfu/day. Following its review, the FDA issued a "no questions" letter on July 31, 2012 (FDA, 2012). Subsequently, the FDA received seven additional GRAS notices on the use of *B. coagulans* in foods and all these GRAS notices received no questions letter from the agency. The details of all these notices are provided in Table 6. In addition to the above mentioned eight GRAS notices, FDA also received two GRAS notices (GRN, 240 and GRN 378) in which the use of *B. coagulans* along with other bacteria, to culture or ferment the food product has been proposed.

**Table 6. FDA Evaluated GRAS Notices on Use of *Bacillus coagulans* in Food**

GRN No.	Substance	Date of closure	FDA's Letter
725	Inactivated <i>Bacillus coagulans</i> GBI-30, 6086	Feb 12, 2018	FDA has no questions
691	<i>Bacillus coagulans</i> SANK 70258 spore preparation	Aug 28, 2017	FDA has no questions
670	Inactivated <i>Bacillus coagulans</i> GBI-30, 6086	Mar 15, 2017	FDA has no questions
660	<i>Bacillus coagulans</i> GBI-30, 6086	Jan 13, 2017	FDA has no questions
601	<i>Bacillus coagulans</i> SBC37-01 spore preparation	Apr 28, 2016	FDA has no questions
597	<i>Bacillus coagulans</i> SNZ 1969 spores preparation	Feb 29, 2016	FDA has no questions
526	<i>Bacillus coagulans</i> strain Unique IS2 spores preparation	Mar 23, 2015	FDA has no questions
399	Preparation of <i>Bacillus coagulans</i> strain GBI-30, 6086 spores	Jul 31, 2012	FDA has no questions
378	Cultured [dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources] fermented by [ <i>Streptococcus thermophilus</i> , <i>Bacillus coagulans</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus bulgaricus</i> and <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i> or mixtures of these strains]	Mar 26, 2012	FDA has no questions
240	Corn, cane, or beet sugar cultured with <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> , <i>Bacillus coagulans</i> LA-1, or <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i> , or mixtures of these microorganisms	Oct 24, 2008	FDA has no questions

Adapted from FDA GRAS Inventory website

Among the eight notices to FDA, the GRAS notice on *B. coagulans* SNZ 1969 spore preparation (GRN 597) for its uses in several conventional foods was submitted by Sanzyme. Following completion of its review, on February 29, 2016, the FDA issued a no question letter on the use of *B. coagulans* SNZ 1969 spore preparation in foods. The subject of the present GRAS assessment for the use of *B. coagulans* SNZ 1969 in infant formula is the same as that of GRN 597. Similarly, the subject of GRN 691, *B. coagulans* SANK 70258 is the mother strain of the subject of the present GRAS assessment. Of the eight GRAS notices that received no question letter from FDA, two notices (GRN 660 and GRN 725), one on *B. coagulans* spore and other on inactivated *B. coagulans*, were for uses in infant formula, respectively. The proposed

use in both these GRAS notices was as an ingredient in non-exempt term infant formula at levels up to  $2 \times 10^8$  cfu per 100 ml of infant formula as consumed. The FDA reviewed these GRAS notices (Table 6), including the two notices for use of *B. coagulans* in infant formula; these notices are incorporated herein by reference.

## 6.2. Safety Related Studies

In recent years, the safety of different strains of *B. coagulans* has been extensively investigated in pre-clinical and clinical studies. The animal toxicity studies of *B. coagulans* include, acute, subchronic and chronic oral toxicity, and one-generation reproduction toxicity. In human clinical studies, in addition to efficacy, relevant safety endpoints were also included in some studies. In addition to human clinical trials in adults, in some randomized, placebo-controlled clinical trials, the effects of *B. coagulans* in infants were investigated. In addition to pre-clinical and clinical studies, the whole genome sequence of *B. coagulans* SNZ 1969 has been screened for the presence of toxicity or pathogenicity related genes. For the present GRAS assessment, all of these studies and information is reviewed as part of the safety evaluation. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. In a majority of the early studies, the form of *B. coagulans* used was not clear. However, it is likely, that in these studies *B. coagulans* in the endospore form has been used.

The safety of the proposed use of *B. coagulans* SNZ 1969 in infant formula was evaluated based on a review of the totality of the available evidence on identification of the microorganism using conventional phenotypic analysis in combination with genotypic analysis, antibiotic resistance of the strain and potential production of virulence factors, potential for toxicity as evaluated in pre-clinical studies, and potential for adverse effects as evaluated in clinical studies for different *B. coagulans* strains. *B. coagulans* is classified as Biosafety Level 1 (BSL-1) organism, thus indicating that the organism is not known to cause disease in healthy human adults. The safety-in-use assessment of *B. coagulans* SNZ 1969 included evidence and data provided in eight GRAS notices (Table 6) on the use of different strains of *B. coagulans* in conventional foods and infant formula. Additionally, more recent information pertinent to the safety of *B. coagulans* identified from searches of the publicly available literature, and a review of data and information on the phenotypic and genotypic analysis of *B. coagulans* SNZ 1969 was evaluated. The totality of evidence, in combination with information on the established history of use of the *B. coagulans*, was relied upon to conclude the safety-in-use.

### 6.2.1. Studies in Infants and Children

In the published literature, some randomized controlled trials with *Bacillus coagulans* in infants were found. As described below, these trials did not reveal any treatment-related adverse effects. Findings from some of these clinical trials related to safety of *B. coagulans* are summarized below and in Table 7. Among the studies described below, only one study (Dutta et al., 2011) has specifically mentioned use of spores. It is likely that in all other studies the spores are used, as *B. coagulans* is primarily available and used in spore forms.

Dhongade and Anjaneyulu (1977) investigated the effects of *B. coagulans* spore preparation (Sporlac- the subject of present GRAS assessment) in the treatment of neonatal diarrhea. A total number of cases selected for the study were 66 (36 males; 30 females) of which 60 were of neonatal diarrhea (watery stools with more than 6 frequency). The authors reported that cases of neonatal diarrhea (n=60) were treated with  $1.5 \times 10^7$  *B. coagulans* spores/day (Sporlac). The duration of treatment was not mentioned in the publication; however, it can be



assumed that the treatment continued until recovery or for a few days. In this study, of the 60 subjects treated, 49 responded within two days of treatment. Based on the dosage level suggested for Sporlac of  $5 \times 10^6$  spores/kg bw, each neonate received approximately  $1.5 \times 10^7$  spores/day. No adverse effects were reported. The investigators stated that Sporlac has been very safe and efficacious in the treatment of neonatal diarrhea. Additional details of study were not available.

**Table 7. Clinical Studies with *Bacillus coagulans* in Infants and Children**

<i>B. coagulans</i> strain	Study type	Study population (age and sex, # randomized [completed])	Duration of intake	Dose; cfu/day	Findings	Reference
<i>B. coagulans</i> <sup>a</sup>	RCT 2 groups	Children with acute watery diarrhea and 'some' dehydration $12 \pm 4$ y (treatment), $11 \pm 4$ y (control); M/F n=80 [78] treatment, n=80 [70]	5 day	$0.24 \times 10^9$	No adverse event or complication was observed	Dutta et al. (2011)
<i>B. coagulans</i> <sup>a</sup>	RCT 2 groups	Preterm infants with a gestational age of <33 week or birth weight of <1500 g 2 d; M/F n=121 [110] treatment, n=121 [111] control	34.5 or 30 days (median)	$0.35 \times 10^9$	The incidence of sepsis did not significantly differ between groups. Other adverse effects attributed to <i>L. sporogenes</i> (flatulence, diarrhea) were not observed	Sari et al. (2014)
<i>B. coagulans</i> <sup>a</sup>	RCT 2 groups	Full-term healthy newborn infants; M/F n=55 treatment, n=57 control	1 year	$0.1 \times 10^9$	No mention of adverse effects	Chandra (2002)

<sup>a</sup> Referred in the publication as *Lactobacillus sporogenes*, a previous name of *B. coagulans*  
Abbreviations: cfu = colony forming units; F = female; M = male;

In another study conducted by Deodhar (1984), the effects of Sporlac (*Bacillus coagulans* SNZ 1969) was studied in neonates with acute gastroenteritis. In this study, 50 neonates (26 males; 24 females; majority 3-6 day old) with transitional diarrhea were selected. All neonates received *B. coagulans* at a dose of 5 million spores per kg body weight per day for 5 days. Improvement was noted in 94% of patients within 1-3 days of treatment. The investigators reported that there were no adverse effects of *B. coagulans* and it is very safe. Additional details of study were not available.

In a double-blind, randomized, placebo-controlled, hospital-based clinical trial, Dutta et al. (2011) investigated the effects of *Bacillus coagulans* (also named in the publication as *L. sporogenes*) against dehydrating diarrhea in children. In this study, children aged 6-24 months who had diarrhea with some dehydration participated. Study participants received tablets containing *B. coagulans* or placebo (control group) and oral rehydration salt solution for correction of initial dehydration as well as maintenance therapy. Duration, frequency, volume of diarrhea and intake of ORS of two groups were compared as outcome variables. In this study, 148 children participated, of whom 78 (Study group) received *B. coagulans* and 70 received placebo (Control group). Subjects received  $1.2 \times 10^8$  cfu (total daily dose  $2.4 \times 10^8$  daily) or

placebo twice daily for up to five consecutive days or recovery, whichever occurred first. Differences in recovery rate, duration, frequency, volume of diarrhea, intake of ORS and other fluids were not significant between both groups. No adverse events or complications were observed during the treatment period or the 15-day no-treatment follow-up period. The product used in this study is likely to be subject of the present GRAS assessment.

In a prospective, blinded, randomized controlled trial, Sari et al. (2011) investigated the effects of orally administered *B. coagulans* (in the publication the name is reported as *Lactobacillus sporogenes*- the old name) in reducing the incidence and severity of necrotizing enterocolitis (NEC) in very low-birth weight infants. The study was conducted in preterm infants with a gestational age of <33 weeks or birth weight of <1500 g. In this study, infants who survived to start enteral feeding were randomized into two groups. The infants in the study group were given *B. coagulans* with a dose of  $3.5 \times 10^8$  cfu mixed in breast milk or mixed feeding (breast milk and formula) once a day, starting with the first feed until discharged. The control group received breast milk or formula only (as the addition of treatment powder did not change the appearance of the test item, which was provided to subjects' caregivers under a blinded protocol). In this study, a total of 221 infants completed the study: 110 in the study group and 111 in the control group. The study lasted for 34.5 or 30 days (median). There was no significant difference in the incidence of death or NEC between the groups. No adverse events attributable to the test item occurred during the study. Feeding intolerance was significantly lower in the treatment group than in the control group. In both, the active treatment and control groups, sepsis occurred with similar incidence; the pathogens were mostly catheter-related and *B. coagulans* was not found in any of the cultures. The findings from this support safe use of *B. coagulans* in infants.

In an article published in the Italian language, La Rosa et al. (2003) investigated the effects of *B. coagulans* (the authors mentioned as *L. sporogenes*) and fructo-oligosaccharides (prebiotic/probiotic) preparation in the prevention of diarrhea due to antibiotics in childhood. In this randomized, double-blind, placebo-controlled trial, a total of 120 children, with active infections requiring antibiotics, were divided into two groups (60/group). In this study, less than 10% of the 120 pediatric subjects were under the age of 2 years with the youngest subject being 4 months old; however, the number of subjects that were infants was not reported. The children were treated orally with the probiotic/prebiotic preparation or a placebo (without prebiotic/probiotic). Children in the treatment group received daily mixture containing *B. coagulans* ( $5.5 \times 10^8$  cfu) and fructo-oligosaccharide (250 mg). The control group (n=60) received the placebo. The patients' diary and follow-up clinical examinations were used to monitor the changes. Of the 98 evaluable subjects, 71% in the group receiving the prebiotic/probiotic treatment had no diarrhea versus 38% in the placebo group. The duration of diarrhea in the treatment group was significantly lower (0.7 days) as compared to the placebo group (1.6 days). The study authors concluded that prophylaxis with the prebiotic/probiotic treatment significantly reduced the number of days and duration of events in children with antibiotic-induced diarrhea. No adverse effects of treatment were reported. The study was conducted in a population that was not well defined and using a test article that was not well defined. The findings from this study corroborate the safety of *B. coagulans*.

In a cross-over, randomized, double-blind, placebo-controlled study, Labalestra et al. (2008) evaluated the effect of a combination of symethicone and *B. coagulans* (Colinox) on the gastric emptying time (GET) and relief of symptoms in infants with symptomatic

gastroesophageal reflux (GER). In this study, 19 children, younger than one year (11 female, 8 male; mean age: 5.5 months) suffering from symptomatic GER were administered with either a combination of oral solution or placebo (4-times daily) for seven days. Wash out period for this study was seven days. In the group receiving the combination of simethicone and *B. coagulans*, GET (min) was shorter compared to placebo. In the group receiving the combination of simethicone and *B. coagulans*, improvement in GER was observed compared to placebo. Adverse events were not reported.

In a double-blind, randomized trial, Chandra (2002) investigated the effects of *B. coagulans* (mentioned in the publication as *L. sporogenes*) on the severity of acute rotavirus diarrhea. The study was conducted in 112 newborn healthy term infants. These infants in rural India were administered daily with an oral dose of either  $1 \times 10^8$  spores *B. coagulans* (n=55) or placebo (n=57). The study was conducted for 12 months. The children were monitored for episodes of rotavirus diarrhea during the course of the study. Administration of *B. coagulans* to infants decreased the episodes of rotavirus diarrhea and also reduced the duration of each episode with decreases in the number of days ill per year (13 days ill in the *B. coagulans* group as compared to 35 days in the control). The investigators did not report any adverse effects, withdrawals, or loss to follow. At one year, a trend for body weight to be higher in the treated group was noted. This was not statistically significant. The results of this study support the safe use of *B. coagulans* in infants. In the publication, the name of source material provider was not mentioned. As the study was conducted in India- the strain used in this study is most likely to be SNZ 1969; locally available.

In summary, in the published literature, three randomized, blinded, placebo-controlled clinical trials in which infants received *B. coagulans*, were located. None of the trials reported any treatment-related adverse effects of *B. coagulans*. In the pre-term infants, administration of *B. coagulans* at dose levels of  $0.35 \times 10^9$  cfu/day for 30 days did not show any adverse effects related to *B. coagulans*. The additional information from other clinical studies corroborate the safety *B. coagulans*.

#### **6.2.2. Human Studies with Lacbon (Mother strain of SNZ 1969)**

The Japanese Ministry of Health and Welfare has approved a product containing *B. coagulans* under the brand name Lacbon (also marketed in India under the name Sporlac). This product is marketed for improvement of symptoms caused by abnormalities in intestinal flora or by dysbiosis. The *B. coagulans* strain present in this product is the mother strain of the subject of the present GRAS assessment. In Japan, clinical trials with Lacbon have been conducted at 19 independent health care institutes. In these trails, Lacbon containing *B. coagulans* at dose levels ranging from  $0.5 \times 10^8$  to  $7.5 \times 10^8$  cfu/day was administered to 567 subjects for 2 to 20 days (Majeed and Prakash 1998, Losada and Olleros 2002). The result from these studies suggest that *B. coagulans* is effective in treating diarrhea for acute or chronic gastroenteritis, mal-digestion, infantile diarrhea and constipation. Adverse events were not reported. Additional details of these investigations were not available for independent review. Some of the findings from the clinical trials studies with Lacbon are presented in Table 8 (Losada and Olleros, 2002). In a review article on the influence of fructo-oligosaccharides and lactobacilli on intestinal health, Losada and Olleros (2002) noted the utility and advantages of *B. coagulans* along with a high degree of safety.

**Table 8: Summary of Results of Different Human Clinical Trials with Lacbon (*B. coagulans*)**

No. of subjects	Condition	Treatment	Findings
38	Acute and chronic intestinal catarrh	$1 \times 10^8$ - $6 \times 10^8$ spores/day for 2-12 days	87% recovery from diarrhea to regular normal stools
15	Diarrhea	$0.75 \times 10^8$ - $6 \times 10^8$ spores/day for 3-12 days	100% recovery from diarrhea to regular normal stools from third to fourth day
10	Constipation	$3 \times 10^8$ - $7.5 \times 10^8$ spores/day for 2-10 days	70% recovery to normal stools and disappearance of abdominal distention
9	Abnormal intestinal fermentation	$1 \times 10^8$ - $6 \times 10^8$ spores/day for 3-14 days	Disappearance of vomiting and nausea in all subject; appetite improved; stools became regular and normal; diarrhea and stomach pain relieved
26	Dyspepsia infantum	$1 \times 10^8$ - $2 \times 10^8$ spores/day for 2-12 days	86% response; general condition and nature of stool improved; frequency of stool decreased to half or less than that before treatment
5	Allergic skin disease	$2 \times 10^8$ - $4.5 \times 10^8$ spores/day for 4-12 days	80% response; obvious eruptions of strophulus and eczema decreased from the third day (topical therapy employed concomitantly)
10	Miscellaneous symptoms	$0.2 \times 10^8$ - $0.5 \times 10^8$ spores/day for 4-20 days	80% response seen in anorexia of nervous type and malnutrition in infants

Adapted from Losada and Olleros (2002)

### 6.2.3. Studies in Adult Human Subjects

Some of the relevant findings from human clinical studies in adult subjects with different strains of *B. coagulans* are summarized in Table 9.

#### 6.2.3.1. Fate in the Human GI Tract

Following oral ingestion, spores of *B. coagulans* passes through the stomach and reach the duodenum, where it germinates and multiplies rapidly (Losada and Olleros, 2002). Upon oral ingestions, spores takes 4 hours to travel to the duodenum or small intestine and start germination. After reaching the intestine, it continues to germinate and becomes metabolically active as a part of facultative anaerobes (capable of producing energy through aerobic respiration and then switching back to anaerobic respiration depending on the amounts of oxygen and fermentable material in the environment) and produces lactic acid fermentation products. *B. coagulans* has been reported to stay temporarily in the human intestinal tract (Majeed and Prakash, 1998). *B. coagulans* are excreted via feces for seven days after discontinuation of administration of *B. coagulans* (Majeed and Prakash, 1998). The evidence suggest that *B. coagulans* improves gastrointestinal ecology by replenishing the quality of desirable obligatory bacteria and antagonizing pathogenic microorganism (Anonymous, 2002).

The effects of daily administration of *B. coagulans* spores ( $2.5 \times 10^8$ /day) for 10 days to a subject on the growth and proliferation of *B. coagulans* in the GI tract were investigated. On the eighth day of administration, the total number of *B. coagulans* remaining in the intestine was  $2.5 \times 10^5$  cfu. On day six, after discontinuation of the treatment, less than ten *B. coagulans* spores were recovered in the feces. The study was repeated, with increasing the dose to  $8 \times 10^8$  spores/day of *B. coagulans* for four days. No *B. coagulans* spores were found in the feces before the administration. By the second day of administration,  $3.8 \times 10^5$  *B. coagulans* spores were found in the feces. On day three after the discontinuation of *B. coagulans* ingestion, there were  $1.1 \times 10^5$

spores in the feces, while on day eight no *B. coagulans* spores were noted in the feces. The results of this study suggest that *B. coagulans* is transiently maintained in the intestinal tract.

#### 6.2.3.2. Clinical Studies in Adults

In a meta-analysis of clinical trials, Doron et al. (2008) reported that the probiotics are used in prevention of antibiotic associated diarrhea. Among the probiotics, *B. coagulans* are most effective and safe. In another review article, Johnston et al. (2007) described prevention of antibiotic associated diarrhea in children by the probiotic together with adverse effects. In this assessment of 10 clinical trials, *Lactobacilli* spp., *Bifidobacterium* spp., *Streptococcus* spp., or *Saccharomyces boulardii* alone or in combination, *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at  $0.5 \times 10^{10}$  to  $4 \times 10^{10}$  cfu/day. *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at 5 to 40 billion cfu/day were found as the most promising probiotics.

In a recent double blind, placebo controlled, multi-centered trial, Majeed et al. (2016) evaluated the safety and efficacy of *B. coagulans* MTCC 5856 in diarrhea predominant IBS patients. In this study, 30 newly diagnosed diarrhea predominant IBS patients were enrolled. Along with standard care of treatment, 18 patients in group one received placebo while in group two 18 patients received a *B. coagulans* MTCC 5856 tablet containing  $2 \times 10^9$  cfu/day as active for 90 days. In addition to efficacy, other parameters were studied. Laboratory parameters, anthropometric and vital signs were within the normal clinical range during the 90 days of supplementation in the placebo and *B. coagulans* MTCC 5856 group. No statistically significant changes in clinical chemistry or vital signs were noted. No serious adverse events were reported. One reported adverse event was determined to be unrelated to the study product. Five dropouts (1 treatment, 4 control) were due to personal reasons. Significantly reduced discomfort (bloating, vomiting, diarrhea, stool frequency, abdominal pain) with treatment was noted. The investigators concluded that the *B. coagulans* MTCC 5856, at a dose of  $2 \times 10^9$  cfu/day, along with standard care of treatment was found to be safe and effective in diarrhea predominant IBS patients for 90 days of supplementation.

Mohan et al. (1990a; 1990b) investigated the effects of *B. coagulans* on serum lipid levels in hypercholesterolemic patients in two open label clinical studies. *B. coagulans* spore ( $3.6 \times 10^8$  cfu/day) was administered to 17 patients suffering from type II hyperlipidemia for 12 weeks. Treatment with *B. coagulans* spore resulted in significant reductions in total cholesterol and LDL-cholesterol. There was no change in serum triglyceride concentration. The total cholesterol to HDL cholesterol ratios was reduced by 24%, while the LDL to HDL ratio was decreased by 33%. Total cholesterol to HDL cholesterol and LDL-cholesterol to HDL-cholesterol ratios was improved during the *B. coagulans* treatment. HDL-cholesterol was found to have increased from 43.6 to 46.8 mg/dl. No adverse effect of the treatment was noted. While the change in serum lipid levels on treatment was consistent with regard to total and LDL-cholesterol, it was not so in the case of serum triglycerides and HDL-cholesterol. The *B. coagulans* used in this study is the subject of the present GRAS assessment (*B. coagulans* SNZ 1969).

Iino et al. (1997a, 1997b), in two separate studies, investigated the effects of *B. coagulans* on intestinal microflora. In the first study, the effects of *B. coagulans* on stool color, stool shape, stool frequency, defecation feeling and stool odor in 28 adult healthy Japanese women were studied (Iino et al., 1997a). The subjects ingested one sachet (containing lactose and  $1 \times 10^8$  *B. coagulans* cells/g) per day for two weeks. Improvements in stool properties (color, shape), along with increases in defecation frequency, was noted. No tolerance data was reported and there were

no reports of adverse events. In the second study, Iino et al. (1997b) studied the effects of *B. coagulans* on intestinal flora, decayed products and stool property. In this study, 18 healthy adult women were divided in three groups to receive  $0.2 \times 10^8$ ,  $1.0 \times 10^8$  and  $2.0 \times 10^8$  cells of *B. coagulans* per day for two weeks. No subject complained of gas generation, diarrhea or continuous abdominal pain problems due to ingestion of *B. coagulans*. The investigators suggested that consumption of *B. coagulans* improves the bacterial flora and improves the health of the individuals. No adverse effects of *B. coagulans* were reported. The *B. coagulans* strain used in these investigations is the mother strain of the subject of present GRAS determination.

Ara et al. (2002) investigated the effects of *B. coagulans* in 23 female volunteers aged 20 to 40 years old. These subjects had a tendency for constipation as a result of changes in the intestinal environment. The study was conducted for 12 weeks. Monitoring of the subjects was done four weeks before administration, four weeks during administration of placebo and four weeks during administration of *B. coagulans* ( $1 \times 10^8$  cfu/day). The subjects were asked to keep a diary on defecation frequency and fecal characteristics (fecal shape, color and odor) and skin characteristics (number of comedones). The skin was analyzed by counting the number of skin eruptions every two weeks. Stool defecation frequency was greater on administration of *B. coagulans* compared to before administration. Seventy-two percent of the subjects reported improvement in constipation or diarrhea after intake of *B. coagulans*. No reports of adverse effects or intolerance of the supplements were noted. The *B. coagulans* strain SANK 70258 used in these investigations is the mother strain of the subject of the present GRAS determination, *B. coagulans* SNZ 1969.

Ara et al (2002) also evaluated the effects of *B. coagulans* powder in 20 healthy adults (16 males and 4 females) with a tendency for constipation, on dermal characteristics as a result of the changes in the intestinal environment. The study duration was six weeks. Monitoring of the subjects was done two weeks prior, two weeks during administration of *B. coagulans* ( $1 \times 10^8$  cfu/day) and two weeks after treatment. Stool samples were collected before administration, 14 days after the start of administration and 14 days after the end of administration. For decomposition products, specimens were analyzed. Defecation frequency and fecal characteristics were examined and recorded from the volunteers. *B. coagulans*, at  $1 \times 10^8$  cfu/day, administration revealed improvement in fecal shape and fecal color from dark brown to yellowish brown, fecal odor decreased, and defecation frequency was increased. Increases in the number of intestinal bifidobacteria was observed. After the administration, intestinal levels of *C. perfringens* was decreased compared to values before intake. Decrease in concentration of intestinal ammonia, indole and p-cresol was observed. The results of this study indicate the improvement in the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics following administration of *B. coagulans*. No reports of adverse effects or intolerance of the supplements were noted while consuming the *B. coagulans* powder. The *B. coagulans* strain SANK 70258 used in this study is the mother strain of the subject of this GRAS determination.

In yet another randomized, placebo controlled, double-blind trial, Kajimoto et al. (2005) investigated the effects of a strain of *B. coagulans* SANK 70258 in subjects suffering from seasonal allergic rhinitis. In this study, 55 volunteers (aged 20-65 years - healthy men and women) with the history of Japanese cedar pollinosis were allocated. The subjects (n=29) randomly received either test food containing  $4 \times 10^8$  viable *B. coagulans* cells or placebo (n=26) for eight weeks. The subjects were monitored for safety related parameters such as hematology

(9 commonly measured parameters) and clinical chemistry (25 commonly analyzed parameters), as well as for improvements from allergy. Gastrointestinal symptoms and skin symptoms were recorded for any adverse effects. The observation from physical examination, hematology and clinical chemistry parameters did not reveal any adverse effects in either of the groups. After intake of the test food, no adverse reactions were noted. The results from this study suggest that *B. coagulans* at a dose of  $4 \times 10^8$  cfu was safe for consumption by humans for eight weeks. The *B. coagulans* SANK 70258 used in this study is the mother strain of the subject of the present GRAS determination.

**Table 9. Human Clinical Studies with Different Strains of *Bacillus coagulans***

<i>B. coagulans</i> strain	Study type	Study population (age and sex, # randomized [completed])	Duration of intake	Dose; cfu/day	Findings	Reference
<i>B. coagulans</i> MTCC 5856	RCT 2 groups	Patients with diarrhea predominant IBS (at least 75% loose or mushy stools) 36.2 ± 11.07 y (treatment), 35.4 ± 10.75 y (control); M/F n=18 [17] treatment, n=18 [14] control	90 day	2x10 <sup>9</sup>	No statistically significant changes in clinical chemistry or vital signs; no serious adverse events; 1 reported adverse event determined to be unrelated to study product. Five dropouts (1 treatment, 4 control) due to personal reasons. Significantly reduced discomfort (bloating, vomiting, diarrhea, stool frequency, abdominal pain) with treatment	Majeed et al. (2016)
<i>B. coagulans</i> <sup>a</sup> + inulin + B-carotene	Crossover 2 groups	Patients with type 2 diabetes 52.9 ± 8.1 y; M/F n=51 [48]	6 weeks	0.3x10 <sup>8</sup>	No serious adverse reactions were reported	Asemi et al. (2015)
<i>B. coagulans</i> + FOS	RCT 3 groups	Children with chronic abdominal pain (>2 months with recurrence at least once per week) 7.44 ± 2.44 y ( <i>B. coagulans</i> + FOS), 7.06 ± 2.38 y (peppermint oil), 7.42 ± 2.49 y (control); M/F n=40 [29] <i>B. coagulans</i> + FOS; n=40 [34] peppermint oil; n=40 [25] control	1 month	0.5x10 <sup>9</sup>	No adverse reactions or intolerance observed	Asgarshirazi et al. (2015)
<i>B. coagulans</i> <sup>a</sup>	RCT 3 groups	Diabetic patients 51.3 ± 10.4 y (synbiotic), 52.0 ± 7.2 y (probiotic), 53.4 ± 7.5 y	8 weeks	0.3x10 <sup>9</sup>	No side effects were reported following the consumption of the probiotic bread	Bahmani et al. (2016); Tajadadi-Ebrahimi et

		(control); M/F (81% F) n=27 [25] synbiotic, n=27 [25] probiotic, n=27 [26] control				al. (2014); Shakeri et al. (2014)
<i>B. coagulans</i> GIB-30, 6086	Crossover 2 groups	Healthy men and women 65-80 y; M/F n=42 [36]	28 day	1x10 <sup>9</sup>	No mention of adverse effects	Nyangale et al. (2015)
<i>B. coagulans</i> lilac-01 + okara powder	RCT 2 groups	Healthy Japanese volunteers with a tendency for constipation 50.6 y; M/F n=148 treatment, 149 control (okara powder) [268]	2 weeks	0.1x10 <sup>9</sup>	No mention of adverse effects	Minamida et al. (2015)
<i>B. coagulans</i> <sup>a</sup> + FOS	RCT 2 groups	Adults with irritable bowel syndrome 39.8 ± 12.7 y; M/F n=41 [23] treatment, n=44 [33] control	12 weeks	0.45x10 <sup>9</sup> FOS – 300 mg/d	17 (41%) patients in the treatment group discontinued the study; 12 (27%) due to vomiting and 5 (12%) due to diarrhea. 11 (25%) patients in the control group discontinued the study; 5 (11%) due to constipation, 3 (7%) due to urticarial, and 3 due to bloating (7%). No other side effects were observed	Rogha et al. (2014)
<i>B. coagulans</i> GIB-30, 6086	RCT 2 groups	HIV infected persons receiving cART Median age 49 y (treatment), 51 y (control); M/F n=12 [10] treatment, n=12 [7] control	90 day	2x10 <sup>9</sup>	No serious adverse events were reported. Only mild gastrointestinal symptoms were reported during the study; in the probiotic group, 3/10 reported bloating. In the placebo group, 1/7 reported increased diarrhea	Yang et al. (2014)
<i>B. coagulans</i> Unique IS-2 (MTCC- 5260)	RCT 2 groups	Women with bacterial vaginosis 32.5 ± 3 y (treatment), 33 ± 3 y (control); F n=20/group	90 day	4x10 <sup>9</sup>	No mention of adverse effects	Sudha et al. (2012a)
<i>B. coagulans</i> Unique IS-2 (MTCC- 5260)	Phase I trial 1 group	Patients with acute diarrhea 35.44 ± 8.76 y; M/F n=28 treatment	10 day	4x10 <sup>9</sup>	Significant reductions in counts of RBC and WBC and serum creatinine levels was observed, however values were within the normal	Sudha et al. (2012b)



					range; no other changes in safety parameters were observed	
<i>B. coagulans</i> Unique IS-2 (MTCC-5260)	Open label 3 groups	Men and women with hyperlipidemia 42-53 y; M/F n=10/group	60 day	10x10 <sup>9</sup> 20x10 <sup>9</sup>	No mention of adverse effects	Sudha et al. (2011b)
<i>B. coagulans</i> GIB-30, 6086	Open label 1 group	Healthy subjects; 27 y; M/F n=10 (10)	28 day	0.5x10 <sup>9</sup>	No serious adverse events were reported throughout the study	Kimmel et al. (2010)
<i>B. coagulans</i> GIB-30, 6086	RCT 2 groups	Patients with symptoms of rheumatoid arthritis 62.5 y; M/F n=23 [22] treatment, n=22 [22] control	60 day	2x10 <sup>9</sup>	No serious adverse reactions reported throughout the study. Treatment group reported 4 adverse events including shingles, poison ivy, a cold and leg edema (all deemed unrelated to study treatment). One subject in the treatment developed an URI and discontinued treatment. Control group reported 3 adverse events including GI reflux, URI, and urinary tract infection	Mandel et al. (2010)
<i>B. coagulans</i> GIB-30, 6086	Crossover 2 groups	Healthy adults; 44 y; M/F n=10 [9]	30 day	2x10 <sup>9</sup>	No serious adverse events were reported throughout the study	Baron et al. (2009)
<i>B. coagulans</i> GIB-30, 6086	RCT 2 groups	Patients with diarrhea prominent IBS 52.3 ± 11 y (treatment), 44.0 ± 17.9 y (control); M/F n=26 [26] treatment, n=29 [26] placebo	8 weeks	2x10 <sup>9</sup>	Adverse events were, for the most part, mild to moderate, and were generally self-limiting. Five patients who received treatment reported 6 adverse events; six patients who received placebo reported six adverse events. One severe adverse event (headache) was reported in the placebo group	Dolin et al. (2009)
<i>B. coagulans</i> GIB-30, 6086	RCT 2 groups	IBS-abdominal pain and bloating patients 48.36 y; M/F n=50 [22/group]	8 weeks	~0.8x10 <sup>9</sup>	No treatment related adverse events or serious adverse events reported during the study	Hun et al. (2009)

					period. Four adverse events reported in the placebo group and two in the treatment group were unrelated to the treatments	
<i>B. coagulans</i> GIB-30, 6086	RCT 2 groups	Patients with self-reported post-meal intestinal gas-related symptoms 34.8 ± 12.5 y (treatment), 38.2 ± 12.6 y (control); M/F n=30 treatment, n=31 control	4 weeks	2x10 <sup>9</sup>	"... the <i>Bacillus coagulans</i> -based probiotic product was effective and safe..."	Kalman et al. (2009)
<i>B. coagulans</i> <sup>a</sup>	RCT 2 groups	Patients with acute or chronic diarrhea 18-65 y; M/F n=103 treatment, n=101 control	3-7 day (acute diarrhea) 14-21 day (chronic diarrhea)	0.3x10 <sup>9</sup>	Body weight, body temperature, respiratory rate, heart rate, blood pressure, blood routine, and liver and renal functions were within normal limits. No treatment related adverse effects	Cui et al. (2004)
<i>B. coagulans</i> <sup>a</sup>	Open label 1 group	Patients with primary hyperlipidemia 45.6 y; M/F n=20 [17]	12 weeks	0.36x10 <sup>9</sup>	No adverse effects were noted except constipation in one patient	Mohan et al. (1990a,b)

Adapted from GRN 691; the strain of *B. coagulans* used in the study is mentioned whenever available

<sup>a</sup> Referred in the paper as *Lactobacillus sporogenes*, a previous name of *B. coagulans*

Abbreviations: cART = combination antiretroviral treatment; CFU = colony forming units; d = day; F = female; FOS = fructo-oligosaccharides; g = gram; GI = gastrointestinal; M = male; mo = month; RBC = red blood cell; URI = upper respiratory infection; WBC = white blood cell

In another randomized, double-blind trial, Cui et al. (2004) investigated the effects of *B. coagulans* in subjects with acute and chronic diarrhea. In this study, 204 subjects were divided into two groups. The control group (n=101) (51 with acute diarrhea and 50 with chronic diarrhea) received tablets containing Golden Bifid (*Bifidobacterium longum*) at a dose of 1 x 10<sup>8</sup> cfu three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea), while the treatment group (n=103) (51 with acute diarrhea and 52 with chronic diarrhea) received *B. coagulans* at a dose of 1 x 10<sup>8</sup> cfu, three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea). No adverse effects were noted in either of the groups. Increases in the number of *Bifidobacterium* and *Lactobacillus* species in the gut was noted in both of the groups. It was concluded that the *B. coagulans* species efficacy and safety are similar to Golden Bifid tablets and is effective in the treatment of acute and chronic diarrhea.

Astegiano et al. (2006) evaluated the effect of a dietary mixture (IBS Active) containing L-tryptophan, inulin, angelica, vegetal charcoal, vitamin PP, group B vitamins (B1, B2, B6) and probiotics (*Bacillus coagulans*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*) in patients suffering with irritable bowel disease. In this study, treatment group (n=37; 11 men and 27 women; mean age 44.3±5.1 years) received IBS Active over a period of 5 to 8 months, while

the control group (n=28; 6 men and 22 women; mean age 48.6±3.7 years) were instructed to continue their customary therapy for 6 months (range, 5-7). Evaluation on subjects was done for abdominal pain and/or distension, constipation, diarrhea, and alternating constipation and diarrhea. Compared with the baseline values, the reduction in abdominal pain in the treatment group was 62%, 55% in abdominal distension, 58% in constipation, 33% in diarrhea, and 62% in alternation constipation and diarrhea. Compared with the baseline values, no statistically significant reduction in symptoms was found in the control group. Post-treatment comparison between the two groups showed that the study product had reduced symptoms and that the difference was statistically significant for abdominal pain, abdominal distension and constipation. The investigators concluded that the use of IBS Active led to a significant improvement in pain symptoms, abdominal distension and regulation of bowel movement in IBS patients. No adverse event were reported.

In a prospective, randomized double-blind, placebo-controlled trial, Kalman et al. (2009) investigated the effect of *B. coagulans* on gastrointestinal symptoms in adults with post-prandial intestinal gas-related symptoms (abdominal pain, distention, flatulence) but no gastrointestinal (GI) diagnoses to explain the symptoms. In this study, 61 adult volunteers aged 36.5±12.6 years (weight 75.4±17.3 kg) received either *B. coagulans* GBI-30 (n=30) or placebo (n=31) for four weeks. In the treatment group, the subjects received one capsule containing 2.0x10<sup>9</sup> cfu *B. coagulans*/day for four weeks. The subjects were evaluated every two weeks. During each visit, the participants were evaluated with a series of questionnaires in addition to hemodynamics (standard biochemical safety testing) and adverse event monitoring. In the publication, the details of the hemodynamic or biochemical parameters were not mentioned. The investigators concluded that the product containing *B. coagulans* was effective in improving the quality of life and reduce gastrointestinal symptoms in adults with post prandial intestinal gas-related symptoms with no GI diagnosis.

In a randomized, double-blind, placebo-controlled trial, Dolin (2009) investigated the effects of the *B. coagulans* preparation on symptoms of diarrhea-predominant irritable bowel syndrome. In this study, 55 volunteers (including patients with diarrhea-predominant irritable bowel syndrome-IBS-D) were divided into two groups. 26 volunteers (7 male, 19 female) received *B. coagulans* (GBI-30, 6086) and 29 volunteers (6 male, 23 female) received placebo once a day for 8 weeks. The patients were advised to take the capsules containing *B. coagulans* or placebo daily for 8 weeks. Adverse events reported for the most part were mild to moderate and self-limiting. Five subjects receiving *B. coagulans* and six receiving placebo reported six adverse effects. In the placebo group, headache as severe side effects were reported. The results suggest that in patients with IBS-D, *B. coagulans* (GBI-30, 6086) is safe and effective in reducing daily bowel movement.

In summary, in over 20 published clinical studies, the effects of *B. coagulans* has been investigated. In these studies there were no reports of serious adverse effects or observed safety concerns. The daily intake of *B. coagulans* was up to approximately 20x10<sup>9</sup> cfu/day and the period of intervention ranged from a few days to approximately 13 weeks. In some studies, reports of mild to moderate gastrointestinal symptoms with intake of *B. coagulans* were noted. However, the effects were generally self-limiting and reversible. Overall, findings from these studies, in both healthy and compromised individuals, did not reveal any evidence of pathogenicity or toxicity following ingestion of *B. coagulans*. In these studies *B. coagulans* was generally well tolerated.

### 6.2.3.3. Reports of Infection and *B. coagulans*

It is well recognized that lactic acid-producing bacteria are non-pathogenic to humans (Fooks and Gibson, 2002; Doron and Gorbach, 2006). Lactic acid bacteria that occur naturally have an excellent safety profile. In spite of their widespread uses, no major safety issues or health risks to humans have been noted (Holzapfel et al., 1995; Salminen et al., 1996). As compared to most of the common *Lactobacillus* and *Bifidobacterium* species, commonly sold at health food stores and/or used in the production of cultured dairy products, *B. coagulans* has a longer safe history of use. The available information from the published studies did not reveal any significant pathogenic or opportunistic illness caused following administration of *B. coagulans*.

Based on the University of Maryland Cancer Center records, Banerjee et al. (1998) reported that 18 febrile patients experienced 24 episodes of *Bacillus* bacteremia from January 1978 to June 1986. In one episode, the cause was identified as related to *B. coagulans*. Twelve of the 24 episodes of *Bacillus* bacteremia were considered possible infections. Of the twelve patients, 4 had clinically documented sites of infection at the time of the bacteremic episodes, but specific microbiologic documentation of the offending pathogen(s) was not obtained. The remaining eight patients did not have a clear cause for the *Bacillus* bacteremia, nor had a clinical site of infection. Therefore, *B. coagulans* is likely only an opportunistic bacteria, and as such, indicates that *B. coagulans* may only be opportunistic in a highly immuno-compromised population, and would not be defined as virulent. No information, in the published literature was found indicating that *B. coagulans* causes infection following oral ingestion.

### 6.2.4. Safety Based on Genome Sequencing

As mentioned earlier, in an unpublished report, a bioinformatics safety assessment of *B. coagulans* SNZ 1969 was carried out based on the whole genome sequence (Heikkinen, 2017). In an attempt to screen for antimicrobial resistance genes (AMR) and virulence factors in *B. coagulans* SNZ 1969, the contigs were annotated using the PATRIC (<https://www.patricbrc.org/genome>) annotation pipeline using RAST (Rapid Annotation using Subsystem Technology), which includes an up-to-date collection of antimicrobial resistance genes and virulence factors (Wattam et al., 2017). In addition, homology searches (Blastn, Blastx or Megablast) of *B. coagulans* contigs were performed against in-house antibiotic resistance gene consisting of a set of resistance gene or protein sequences from public databases (CARD, ARDB, Redfinder, Genebank bioproject 313047). For protein searches, 80% identity and coverage were used as threshold values. The following two hits to antimicrobial resistance genes were found: (1). Undecaprenyldiphosphatase (UppP; EC 3.6.1.27) (identity 97%) and (2). Translation elongation factor Tu (identity 86%). Among enterococci, susceptibility or resistance to rifamycins appears to be determined by elongation factor EF-Tu (Miele et al., 1994).

UppP is involved in bacitracin resistance as bacitracin supposedly sequesters undecaprenyl disphosphate (UppP enzyme substrate) which reduces the pool of lipid carrier available to the cell (El Ghachi et al., 2004). Bacitracin is not of human or veterinary importance. According to the CARD database, the mutations conferring resistance are G258A and G275A. The protein was found with 86% identity from *B. coagulans*, but the mutations were not detected. This gene is of no concern and the potential resistance is not of human or veterinary importance (Heikkinen, 2017).

As regards putative virulence factors the following matches were found: (1) ATP-dependent Clp protease ATP-binding subunit ClpX (identity 85%), (2) GTP-sensing transcriptional pleiotropic repressor codY (identity 81%), (3) Adenylosuccinate synthetase (EC 6.3.4.4) (identity 83%); and (4) Adenylosuccinate lyase (EC 4.3.2.2) @ SAICAR lyase (EC 4.3.2.2) (identity 80%). ClpX is the regulatory ATPase subunit of the ClpXP protease, and contributes to innate defense peptide resistance (McGillivray et al., 2009). The activity of CodY is required for full virulence of *B. anthracis* (Van Schaik et al., 2009). Adenylosuccinate synthetase was identified to be required for intracellular replication of *Listeria monocytogenes* within epithelial cells (Schauer et al., 2010). Adenylosuccinate lyase was demonstrated to be important for *Listeria monocytogenes* colonizing the gastrointestinal tract and cause systemic infection of the spleen, liver and gallbladder on A/J mice (Faith et al., 2012). However, cytotoxicity tests did not show any cytotoxicity for *B. coagulans*. Additionally, enterotoxin genes *nhe*, *hbl* and *cytk*, and cereulide synthase were searched from the genome. No indication of enterotoxins were found (Heikkinen, 2017).

In summary, genes known to confer resistance to antibiotics relevant to human or veterinary importance were not found in the genomic sequences of *B. coagulans* SNZ 1969. Neither were toxin genes or hazardous virulence factors found in the genome (Heikkinen, 2017).

As indicated earlier, Khatri et al. (2016) also analyzed the genome of *B. coagulans* SNZ 1969 and performed comparative analysis at the genomic level for any safety concern. These investigators reported that the domain Fibronectin-binding protein (FBP), N-terminal (PF07299), which is predicted to play a role in virulence in *Listeria*, was absent in the *B. coagulans* genome. Antibiotic resistance proteins were identified by subjecting the proteomes of *B. coagulans* to hmmscan against the Resfams database and no evidence for the presence of plasmids in *B. coagulans* was found, thus the gain and transfer of any plasmid-borne antibiotic-resistance genes are not obvious at this stage. Nevertheless, Khatri et al. (2016) searched for the presence of antibiotic resistance genes and related efflux pumps in the chromosomes. Analysis of antibiotic resistance genes indicated that larger numbers of major facilitator superfamily (MFS) and other efflux transporters are present in *Bacillus*. Tetracycline MFS efflux and class A and class D domains of  $\beta$ -lactamases were found to be absent from *B. coagulans*. Antibiotic inactivation mechanism was absent in *B. coagulans*.

Antibiotic-resistance in a strain can develop due to random mutation or genetic acquisition, and appropriate selective evolutionary pressure (e.g., sub-lethal concentrations of antibiotics present in culture media). *B. coagulans* SNZ 1969 strain is maintained in pure culture, kept frozen and without the presence of antibiotics. As such, it is unlikely that additional antibiotic resistance has been acquired by the *B. coagulans* SNZ 1969.

#### **6.2.5. Specific Antibiotic Susceptibility Studies**

In an unpublished *in vitro* study, Heikkinen (2017a) investigated the antibiotic susceptibility of *B. coagulans* SNZ 1969 to several commonly used antibiotics such as gentamicin, kanamycin, neomycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, vancomycin, quinupristin-dalfopristin, ciprofloxacin, linezolid and rifampicin. The study was conducted in accordance with ISO20776:2006 standard with VetMIC Lact-1 and VetMIC Lact-2 plates (SVA National Veterinary Institute, Uppsala, Sweden) in aerobic conditions, at +35 °C for 18 hours using Mueller Hinton Broth (LabM, LAB114). Additionally, antibiotic susceptibility of *B. coagulans* SNZ 1969 to sulfamethoxazole and bacitracin was

analyzed according to CLSI M07-10 with agar plate dilution method in aerobic conditions, at +35 °C for 18 hours using Mueller Hinton Broth (LabM, LAB114) and Mueller Hinton Agar (LabM, LAB114 and MC006).

The antibiotic susceptibility was evaluated by measuring the minimum inhibitory concentration (MIC) values. In order to distinguish resistant from susceptible strains, the EFSA Panel has suggested microbiological cut-off values (EFSA 2012a). The MICs for *B. coagulans* SNZ 1969 are compared with MIC cut-off values reported by EFSA (2012a) in Table 10. The findings from antibiotic susceptibility assay showed that none of the MIC-values for *B. coagulans* SNZ 1969 exceeded the EFSA MIC cut-off values.

**Table 10: Comparison of Minimum Inhibitory Concentrations (MIC) Values of Antibiotics for *B. Coagulans* SNZ 1969 with EFSA Values**

Antibiotic Studied	MIC (µg/ml)	EFSA MIC Cut-off (µg/ml)
Gentamicin	<0.5	4
Kanamycin	<2	8
Neomycin	<0.5	-
Streptomycin	<0.5	8
Tetracycline	<0.12	8
Erythromycin	<0.016	4
Clindamycin	<0.03	4
Chloramphenicol	2	8
Vancomycin	<0.25	4
Quinupristin-dalfopristin	0.5	-
Ciprofloxacin	<0.25	-
Linezolid	0.5	-
Rifampicin	0.25	-
Sulfamethoxazole	2	-
Bacitracin	1	-

Hong et al. (2008) described unpublished work from another laboratory in which 33 isolates of *Bacillus* strains were tested and over half showed resistance to clindamycin. Similarly, Sorokulova et al. (2008) also reported clindamycin MIC above the EFSA break-point for other probiotic *Bacillus* strains such as *B. licheniformis*. Hong et al. (2008) speculated that clindamycin resistance may be an intrinsic characteristic of *Bacillus* species.

#### 6.2.6. Specific Antimicrobial Action Studies

In an *in vitro* study, antimicrobial potentials of *B. coagulans* SNZ 1969 against some pathogenic bacteria such as *Salmonella abony*, *Streptococcus faecalis* and *Escherichia coli* were investigated (Ashtekar, 2012; also described in GRN 597). *B. coagulans* SNZ 1969 did not inhibit any of these pathogenic strains, indicating that SNZ 1969 cultures do not produce detectable levels of inhibitory substances against the test cultures. In another *in vitro* study, antimicrobial action of *B. coagulans* SNZ 1969 was investigated against some of the gastrointestinal resident microorganisms such as *Lactobacillus panis*, *L. fermentum* and *L. plantarum*. In this study, none of the test cultures were inhibited, indicating that *B. coagulans* SNZ 1969 cultures do not produce detectable levels of inhibitory substances against the test cultures. Similar results were noted with *B. coagulans* ATCC 7050.

The available information indicates that *B. coagulans* does not produce antibiotics. FDA has listed the use of *B. coagulans* in the production of glucose isomerase enzyme. In its list of enzyme preparations used in food, FDA has stated that, “Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxic microorganisms, including *Streptomyces rubiginosus*, *Actinoplanes missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes* and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic.”<sup>7</sup>

#### **6.2.7. Specific Bile Salt and Acid Tolerance Studies**

The tolerance capacity of *B. coagulans* SNZ 1969 in varying concentrations of bile salts was investigated by Ashtekar (2012; also described in GRN 597). High bile salt percentage solutions were prepared by suspending bile salts [Hi-Media CR 008] at 1, 2 and 3% in a sterile sodium chloride solution (0.5%). In order to check the tolerance of *B. coagulans* strains, turbidity (optical density) of the cultures was hourly monitored at 600 nm. The results revealed that at low salt concentration there was a decrease in the growth of *B. coagulans* SNZ 1969 with time. At higher concentration of bile salts static growth condition is seen for first 2 hours and then there is decrease by the 3<sup>rd</sup> hour. *B. coagulans* SNZ 1969 is initially tolerant to 3% bile salts but with passage of time, it appears to be sensitive.

For acid tolerance capacity, a preculture of *B. coagulans* SNZ 1969 was prepared by inoculating one loop onto PNY medium slants (Hi-media M835) and incubated at 37°C for 24 hours. Overnight cultures were taken and washed off with 3x2 ml 0.5% saline. These cells were suspended in 0.1 N HCl and incubated at 37°C for 3 hours. In order to check the tolerance of *B. coagulans* strain, the viable count of the cultures were monitored for every 30 minutes up to 3 hours. The results of this experiment revealed that *B. coagulans* SNZ 1969 was quite stable in acidic conditions with loss of approximately 20% viability by 2 hours.

#### **6.2.8. Animal Toxicity Studies**

In the published literature, several animal toxicity studies have appeared in which potential adverse effects of different strains of *B. coagulans* have been investigated. A summary of these studies is presented in Table 11.

##### **6.2.5.1 Acute Animal Toxicity Studies**

In an unpublished acute toxicity study, conducted as per OECD Guidelines for the Testing of Chemicals No. 423, the LD<sub>50</sub> of *B. coagulans* SNZ 1969 was investigated in Wistar rats (Kumar, 2018). In this study, female rats were administered orally with a single dose of *B. coagulans* SNZ 1969 (264.2 billion cfu/g) at level of 2000 mg/kg bw). Following the dose administration, the animals were observed for clinical signs at 0.5, 1, 2 and 4 hours and thereafter once daily for 14 consecutive days. On Day 15, the animals were euthanized and subjected to gross pathology. Oral administration of *B. coagulans* SNZ 1969 at the dose level of 2000 mg/kg bw did not induce any mortality or morbidity. No abnormal clinical signs, abnormal body weight changes and gross pathological changes were noted. The results of this study show that the LD<sub>50</sub> of *B. coagulans* (264.2 billion cfu/g) was greater than 2000 mg/kg bw.

<sup>7</sup> Available at:

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm>

In an acute study, *B. coagulans* powder was administered to male rats via gavage at dose levels of 1.3 or 5 g/kg bw. The animals were observed for 7 days. Adverse effects like diarrhea and mortality was not seen. Slight distension of the stomach was seen in a few rats receiving the highest dose (5 g/kg bw). After a few hours, these animals were seen to be recovered. It was observed that the *B. coagulans* LD<sub>50</sub> was greater than 5 g/kg bw (Losada and Olleros, 2002). In another acute toxicity study, *B. coagulans* GBI-30 was administered to Wistar rats at a dose level of 5 g/kg bw ( $5.2 \times 10^{11}$  cfu/kg bw) which did not show any mortality or adverse effects. The results suggest that the LD<sub>50</sub> of *B. coagulans* was greater than 5 g/kg bw (Endres et al., 2009). In another acute oral toxicity, *B. coagulans* Unique IS2 was investigated in Sprague Dawley (SD) rats (6/sex/group) at three doses (0 (control, vehicle), 3250 and 6500 mg/kg bw). The findings of this study after 14 days of observation show that the LD<sub>50</sub> of *B. coagulans* is greater than 6500 mg/kg bw ( $32.5 \times 10^9$  cfu/kg bw) (Sudha et al., 2011a). The available acute oral toxicity studies suggest that the oral LD<sub>50</sub> for different strains of *B. coagulans* ranges from 5000 - 6500 mg/kg bw.

**Table 11. Animal Toxicity Studies with Different Strains of *Bacillus coagulans* (Adapted from GRN 691)**

Reference	Strain of <i>B. coagulans</i>	Study type	Study design (animal, # per group)	Duration	Dose	Results
Gu et al. (2015)	CGMCC 9951	Short-term repeat dose toxicity	KM mice, females: n=6/group; males: n=5/group	28 day	0, $1 \times 10^6$ , $1 \times 10^8$ , $1 \times 10^{10}$ spores/kg bw/day	Investigators concluded safe dose as $1 \times 10^{10}$ spores/kg bw/day
Endres et al. (2011)	GBI-30, 6086	Combined chronic/one generation reproductive study	OECD Guideline 452, HsdBrlHan Wistar rats	1 year, 1 generation	0, 600, 1300 or 2000 mg/kg bw/d of test article of $6.88 \times 10^{10}$ cfu/g	NOEL for parental male and female rats: 2372 and 3558 mg/kg bw/d (mean value), respectively NOEL for reproductive performance of male and female rats: 2372 and 3558 mg/kg bw/day (mean value), respectively NOEL for F1 offspring: 3558 mg/kg bw/day (mean value)
Sudha et al. (2011a)	Unique IS2	Short-term repeat dose toxicity	OECD Guideline 407, SD rats, 6 animals/sex/dose	28 day	0, 130, 650, 1300 mg/kg bw/d orally "fed" from stock test article of $5 \times 10^9$ cfu/g	Authors derived a NOAEL of 1300 mg/kg bw/d based on no findings of toxicity at this dose. All observations noted were considered to be non-significant
Sudha et al. (2011a)	Unique IS2	Acute toxicity	OECD Guideline 401, SD rats, 6/sex/dose	Single, oral dose (gavage implied)	0, 3, 250, and 6500 mg/kg bw/d of test article of $10 \times 10^9$	No treatment related effects were observed at any dose, including no findings of body weight changes, clinical signs, or gross pathological



					cfu/g daily	changes
Endres et al. (2009)	GBI-30, 6086	Acute eye irritation study	New Zealand white rabbits	Single application with observations at 1, 24, 48, and 72 h	0.1 g of undiluted cell mass at $1.93 \times 10^{11}$ cfu/g applied to one eye, second eye served as control, without washing after application	Slight to moderate conjunctival irritant effects observed that were fully reversible after 72 h. No corneal involvement or adverse signs in iris. Would not be classified as eye irritant
Endres et al. (2009)	GBI-30, 6086	Acute eye irritation study	New Zealand white rabbits	Single application of 4 h duration with observations at 1, 24, 48, and 72 h. OECD Guideline 404 compliant	0.5 g of undiluted cell mass at $1.93 \times 10^{11}$ cfu/g moistened with water and applied to 6 cm <sup>2</sup> intact skin, then animals were wrapped	Test results demonstrate that article is not irritating the skin. Slight erythema after 1 h exposure, but all findings were minor and fully reversible
Endres et al. (2009)	GBI-30, 6086	Acute oral toxicity	OECD Guideline 423, with 5 animals/sex/dose, Wistar CrI:(WI) BR rats	Single oral gavage dose	0 or 5000 mg/kg bw in 1% methylcellulose in water (control received vehicle only)	The single dose produced no treatment-related signs of toxicity and no body weight changes in the 14-day post-dose observational period. Gross pathological examination revealed no remarkable differences between treated and control animals
Endres et al. (2009)	GBI-30, 6086	Sub-chronic 13-week oral toxicity	OECD Guideline 408 as well as Red Book guidelines followed with 10 animals/sex/dose, Wistar CrI:(WI) BR rats	13 weeks	0, 100, 300, and 1000 mg/kg bw/d by oral gavage suspension in 1% methylcellulose in water from test article stock of $1.36 \times 10^{11}$ cfu/g	NOAEL considered by authors to be >1000 mg/kg bw/day. Effects observed included some decreases in water consumption, hematology and clinical chemistry alterations, lower absolute brain weights in high dose males, lower relative kidney and adrenal weights in some females, and mean body

						weight in high dose males. All observations were determined by authors to be reflective of individual variability, within historical control ranges, or not biologically significant
--	--	--	--	--	--	--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------

### 6.2.5.2. Repeat-Dose Animal Toxicity Studies

In an unpublished repeat dose toxicity study, conducted as per Guidelines for the Testing of Chemicals No. 407 and in compliance with OECD Principles of Good Laboratory Practices, the effects of *B. coagulans* SNZ 1969 were investigated in rats following oral administration (Lavanya, 2015). In this study, highly concentrated, i.e., undiluted *B. coagulans* cell mass of potency  $1.84 \times 10^{11}$  cfu/g was orally administered (gavage) to Wistar rats (6 animals/sex/group) at doses of 0, 250 (46 billion), 500 (92 billion) and 1000 (184 billion) mg/kg bw/day for 28 consecutive days. The highest dose treated animals received a dose of  $1.84 \times 10^{11}$  cfu *B. coagulans*/kg bw/day.

Following treatment, no mortality was observed at any dose level throughout the treatment period. No treatment related clinical signs or symptoms were observed in any of the animals throughout the study. Detailed clinical examination revealed no evidence of treatment related changes in animals throughout the treatment period. Ophthalmological examination revealed no test item related changes in both the eyes at the end of treatment period from group G1 and group G4. No treatment related changes in body weight were noticed in both males and females throughout the treatment period as compared to the control group of animals. Individual animal feed consumption of the test groups of both the sexes was comparable.

All hematological parameters in animals of different test groups of both the sexes were comparable to their respective control groups. Changes observed in hematological parameters were comparable to baseline values and biologically insignificant and could not be correlated to any of the other toxicological findings. Changes observed in clinical chemistry parameters were comparable to baseline values; hence, these changes were considered to be insignificant and could not be correlated with the effect of test item administration. Urine parameters in the test groups of both sexes were comparable and revealed no significance as compared to the respective control animals. Absolute and relative organs weights in both sexes at all dose groups were found to be comparable to the control group. No treatment related gross pathological lesions were observed in any of the animals euthanized at the end of the treatment period. Histopathological examination revealed no evidence of treatment related changes in the organs/tissues evaluated. Histopathological examination was normal in control and treated animals at comparable levels. Under conditions of this study, it is determined that the No-Observed Adverse Effect Level (NOAEL) of the test item is 1000 mg/kg bw/day ( $1.84 \times 10^{11}$  cfu *B. coagulans*/kg bw/day).

In a subchronic toxicity study conducted in accordance with OECD guidelines, Akagawa et al. (2016) evaluated the toxicological profiles of *B. coagulans* strain SANK70258 in rats. In this repeat-dose oral gavage study, *B. coagulans* ( $5.09 \times 10^{11}$  cfu/g) was administered to 6-week old Crl:CD (SD) rats (10/sex/group) for 90 consecutive days at dose levels of 0, 500, 1000, and

2000 mg/kg bw/day. All standard safety related parameters as per OECD guidelines were investigated. According to the results, no deaths occurred in either males or females, and no treatment-related changes were observed in any of the clinical signs including a detailed observation with functional observational battery (FOB), functional test, motor activity, body weight, food consumption, ophthalmoscopy, urinalysis, hematology, blood chemistry, organ weight, necropsy or histopathology. Some hematological and clinical chemistry parameters did show significant changes in the treatment group as compared to the control group. However, these changes were not considered as treatment related as the occurrence was sporadic, there was no dose-response relationship, and values were within the historical control ranges. In this study there were no increases in WBC, neutrophil or eosinophil, or any histopathologic changes indicative of inflammation in any organ/tissue including the digestive track at the maximum dose, suggesting that the *B. coagulans* did not infect rats. The NOAEL was determined as 2000 mg/kg bw/day, the highest dose tested, in males and females. This dose is equivalent to a daily intake of  $1.02 \times 10^{12}$  cfu/kg bw/day. The investigators concluded that *B. coagulans* is harmless and can be used as a probiotic. The test organism used in this study is the mother strain of the subject of the present GRAS assessment.

In a short-term oral toxicity study, Sudha et al. (2011) investigated the potential adverse effects of *B. coagulans* Unique IS-2 strain in rats. In this study, Sprague Dawley rats were orally (gavage) fed with 0, 130, 650, 1300 mg *B. coagulans* Unique IS2 preparation/kg bw/day for 14 consecutive days and follow up was done for 28 days. The *B. coagulans* Unique IS2 preparation contained  $5 \times 10^9$  cfu/g. No treatment-related changes were observed in clinical signs, body weights, feed intake, urine parameters, hematological examinations, clinical chemistry, gross pathology and histopathology. Based on the results of this study, the investigators concluded that *B. coagulans* Unique IS2 was clinically well tolerated at doses up to 1300 mg or  $6.5 \times 10^9$  cfu/kg bw/day, when administered orally to Sprague Dawley rats for 14 consecutive days. The No Observed Adverse Effect Level (NOAEL) for *B. coagulans* Unique IS2 was determined as 1300 mg ( $6.5 \times 10^9$  cfu)/kg bw/day, the highest dose tested (Sudha et al., 2011).

In short-term repeat-dose studies, dogs (n=2), rabbits (n=3) and guinea pigs (n=15) were orally administered maximum ingestible single daily doses of 10 g/kg bw, 30 g/kg bw and 50 g/kg bw of *B. coagulans* powder preparation, respectively, for 7 days (Losada and Olleros, 2002). During the course of the treatment as well as for 10 days subsequent to the withdrawal of treatment, no adverse effects were noted. In a long-term repeat-dose study, male rats were fed a preparation containing  $5 \times 10^9$  spores of *B. coagulans*/g at levels of 0.3, 3 and 5 g/kg bw/day for 15 months. No differences in body weight gains between treated groups and the control group were noted. As compared to the control group, no significant differences in organ weights were noted in the treated groups. Additional details of these investigations were not available (Sankyo, 1968; cited in Majeed and Prakash, 1998; Anonymous, 2002). Although details of these early experiments are not available, these studies indicate that *B. coagulans* preparation is non-toxic. The *B. coagulans* strain used in these studies is the mother strain of the subject of this GRAS assessment.

In a subchronic toxicity study, Endres et al. (2009) investigated the effects of *B. coagulans* cell mass ( $1.36 \times 10^{11}$  cfu/g) in Wister CrI:(WI) BR rats (10/sex/group) following oral administration at doses of 0, 100, 300 and 1000 mg/kg bw/day for 90 consecutive days. The highest dose received by the animals was  $1.36 \times 10^{11}$  cfu *B. coagulans*/kg bw/day. The study was conducted as per OECD guidelines. The highest dose treated animals received a dose of

$1.36 \times 10^{11}$  cfu *B. coagulans*/kg bw/day. No deaths or treatment-related clinical changes were observed throughout the study period in any of the groups. Appearance and behavior of the animals were similar for all groups. No toxicologically significant differences between the treatment and control groups with respect to feed consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations was noted. At termination, no treatment-related macroscopic or microscopic changes in the organs were noted. The NOAEL for both males and females was determined as  $>1000$  mg ( $1.36 \times 10^{11}$  cfu)/kg bw/day, the highest dose tested (Endres et al., 2009). In addition to the animal toxicity studies, Endres et al. (2009) also investigated the potential genotoxic effects of *B. coagulans* (GBI-30, 6086) in *in vitro* bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, and *in vivo* mammalian micronucleus test. In these studies, *B. coagulans* did not reveal mutagenic, clastogenic, or genotoxic effects.

In a subsequent one year study, Endres et al. (2011) investigated the long term effects of *B. coagulans* consumption in rats. This was a combined study to investigate chronic oral toxicity along with one-generation reproductive toxicity. In this feeding study, Wistar rats (20/sex/group) were maintained on a diet containing *B. coagulans* preparation at levels of 0, 10000, 20000 and 33300 mg/kg feed for 52 to 53 weeks. The equivalent dose level was 0, 600, 1200 and 2000 mg/kg bw/day, respectively. No mortality was noted in the treatment groups. Clinical observations did not reveal any toxic signs related to the test article. No *B. coagulans* treatment-related changes in body weight, body weight gain, or feed consumption were noted during the study. Blood samples drawn at 3 weeks and 3, 6 or 12 months did not reveal any toxicological relevant changes in hematology, clinical chemistry or urinalysis. Statistically significant changes noted were either not dose-related, or were well within the historical background range or not correlated with other hematological or histopathological changes. At termination, macroscopic and microscopic examinations did not reveal any treatment-related lesions. The NOEL in male and female rats was determined as 1948 and 2525 mg/kg bw/day, respectively, the highest dose tested (Endres et al., 2011). The test article contained  $6.88 \times 10^{10}$  cfu/g; therefore, the NOEL is equivalent to  $1.34 \times 10^{11}$  cfu/kg bw/day and  $1.74 \times 10^{11}$  cfu/kg bw/day for male and female rats, respectively.

In the one-generation reproductive toxicity study by Endres et al. (2011), Wistar rats divided in to four groups (males- 10/group; females- 20/group) were fed a diet containing *B. coagulans* preparation at a dose levels of 0, 600, 1200 and 2000 mg/kg bw/day. For this study, male rats were fed the diet for 70 days before mating and during the three-week mating period, while female rats were fed for ten weeks prior to mating, during the three-week mating period, throughout pregnancy and lactation and up to weaning of the F1 offspring (up to weaning; postnatal day 21). No mortality was reported in the parental generation. Pregnancy outcome, reproductive performance and live births were unaffected by the treatment. There were no signs of treatment-related toxicity on the F0 (parental) generation (male or female). The NOEL for the parental group (reproductive performance) male and female rats was established as 2372 and 3558 mg/kg bw/day, respectively. The NOEL for the F1 offspring was determined as 3558 mg/kg bw/day. The test article contained  $6.88 \times 10^{10}$  cfu/g; therefore, the NOEL is equivalent to  $2.45 \times 10^{11}$  cfu/kg bw/day (Endres et al., 2011). The postnatal observations in the F1 generation from this study is of some additional significance to the consideration of use of *B. coagulans* in infant formula.

Cavazzoni et al. (1998) investigated the effects of *B. coagulans* during the first seven weeks of life in chickens. In this study, 75 male Ross strain broiler chickens were randomly assigned to three treatment groups: Group C- received the standard diet without any additive; Group A- received the antibiotic virginiamycin (10 ppm) contained in the daily diet; and Group P- received *B. coagulans* daily at a dose level of  $1.6 \times 10^{10}$  cfu/kg/day (1000 ppm) for the first seven days of life, then fed  $4.0 \times 10^9$  cfu/kg/day (250 ppm) during days 8-49. The investigators noted that *B. coagulans* became integrated in the enteric microflora and did not interfere with other bacterial groups in this animal model. *B. coagulans* was found to be transient, without any adhesion to the intestinal epithelium. Presence of *B. coagulans* was detected in the feces after one week treatment.

In summary, the safety of multiple strains of *B. coagulans* has been examined in a variety of animal toxicity studies. The *B. coagulans* strains tested in these studies are very similar to the subject of the present GRAS assessment. The data from these studies can be used to support the safety of *B. coagulans* SNZ 1969 spores' preparation. In fact, in one study by Akagawa et al. (2016), the mother strain of *B. coagulans* SNZ 1969 was used. Studies of several strains of *B. coagulans* indicate that the organism is not toxic as evidenced from acute toxicity, subchronic toxicity, chronic toxicity (one year) and reproductive toxicity studies. The longest duration oral toxicity study of *B. coagulans* was a one-year repeat dose toxicity study of *B. coagulans* GBI-30, 6086. Based on these studies the lowest NOAEL can be established as 1948 mg/kg bw/day or  $1.34 \times 10^{11}$  cfu/kg bw/day. In the subchronic toxicity study with *B. coagulans* SANK70258 (mother strain of the subject of present GRAS), the NOAEL was determined as 2000 mg/kg bw/day, equivalent to a daily intake of  $1.02 \times 10^{12}$  cfu/kg bw/day. Additionally, the lack of adverse effects during postnatal observations in the F1 generation part of the reproductive study is of additional significance to the consideration of the use of *B. coagulans* in infant formula.

### 6.3. Expert Panel Review, Summary and Discussion

At the request of Sanzyme Biologics, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)<sup>8</sup>, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to evaluate the Generally Recognized As Safe (GRAS) status of a standardized *Bacillus coagulans* SNZ 1969 spore preparation for use as a food ingredient in non-exempt term infant formula (including powder, liquid concentrates, and ready-to-feed formulas) at maximum addition levels up to  $2 \times 10^8$  cfu per 100 ml infant formula as ready for consumption. The subject of the present GRAS notification, *B. coagulans* SNZ 1969 spore preparation, for use in term infant formula is the same as that of Sanzyme Ltd. GRAS notice (GRN 597) that received a "no question letter" from FDA for the use of *Bacillus coagulans* SNZ 1969 spores preparation in conventional foods.

A comprehensive search of the scientific literature for safety and toxicity information on *B. coagulans*, including *B. coagulans* SNZ 1969, and other strains, was conducted through March 2019, and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Sanzyme Biologics and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on May 08, 2019, and unanimously agreed to the decision described herein.

---

<sup>8</sup>Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

Sanzyme Biologics ensured that all reasonable efforts were made to identify and select a balanced Expert Panel with expertise in food safety, toxicology, and microbiology. Efforts were placed on identifying conflicts of interest or relevant ‘appearance issues’ that could potentially bias the outcome of the deliberations of the Expert Panel and no such conflicts of interest or ‘appearance issues’ were identified. The Expert Panel received a reasonable honorarium as compensation for their time; the honoraria provided to the Expert Panel were not contingent upon the outcome of their deliberations.

*B. coagulans* SNZ 1969 proposed for use in non-exempt term infant formula by Sanzyme Biologics is a gram-positive, catalase-positive, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. *B. coagulans* SNZ 1969 is a dark grayish white powder with a slightly sweet taste and characteristic odor. The specifications of the product containing *B. coagulans* SNZ 1969 spores has been fully developed by Sanzyme Biologics. *B. coagulans* SNZ 1969 has been deposited with MTCC under MTCC 5724 and BCCM™/LGM with the assigned number LMG S – 27484. The bacterial strain is an isolate of *B. coagulans* obtained from green malt. The identity of *B. coagulans* SNZ 1969 has been fully investigated and confirmed by phenotypic, genotypic and complete genome sequencing analysis. The available evidence using conventional phenotypic analysis, in combination with genotypic analysis and whole genome sequencing, confirms the identity of the *B. coagulans* SNZ 1969 microorganism as a strain of *B. coagulans*. *B. coagulans* SNZ 1969 is manufactured according to current good manufacturing practices (GMP), by a fermentation process using food grade ingredients. The proposed use of *B. coagulans* SNZ 1969 at maximum use levels of  $2 \times 10^8$  cfu/100 ml of term infant formula as ready for consumption will result in a conservative high-end estimated daily intake of  $4.27 \times 10^8$  cfu *B. coagulans*/kg bw.

*B. coagulans* was first isolated and described in 1932. As *B. coagulans* forms spores, it possesses high heat and acid resistance providing advantages for its use in food as a probiotic. The available information suggest that *B. coagulans* has been in use for over 50 years. *B. coagulans* is used in the production of a protein-rich food known as *ugba* in African countries. This microorganism is also used as a probiotic to improve and maintain ecological balance of the intestinal microflora. *B. coagulans* is marketed as dietary supplement probiotics for human consumption to improve and maintain ecological balance of the intestinal microflora. *B. coagulans* has been used as probiotic for the prevention and treatment of acute diarrhea and intestinal infections, as well as for gastrointestinal side effects due to antibiotic therapy. *B. coagulans* is approved for use in the preparation of enzymes used for food production. As per 21 CFR § 184.1372, insoluble glucose isomerase enzyme produced from *B. coagulans* is recognized as GRAS. The available information suggests that *B. coagulans* is well-tolerated, non-pathogenic and non-toxicogenic and there is a common knowledge of safe use of *B. coagulans* for several decades. *B. coagulans* is classified as BSL-1 organism, thus indicating the organism is not known to cause disease in healthy human adults.

The safety of *B. coagulans* SNZ 1969 is supported by whole genome sequence analysis. For this analysis, all coding sequences were examined, including those of potential safety concern. The available information from genome sequencing analysis suggest that *B. coagulans* SNZ 1969 is sensitive to antibiotics and does not harbour any resistance genes that might be transferred. The genes known to confer resistance to antibiotics relevant to human or veterinary importance were not found in the genomic sequences of *B. coagulans* SNZ 1969. Neither were toxin genes or hazardous virulence factors found in the genome.

In the published three randomized, blinded, placebo-controlled clinical trials, administration of *B. coagulans* to infants did not reveal any treatment-related adverse effects. In one study, pre-term infants received *B. coagulans* at dose levels of  $0.35 \times 10^9$  cfu/day for 30 days. In this and other studies in children no adverse effects related to *B. coagulans* were noted. In addition to the studies in infants, the effects of different strains of *B. coagulans* have been investigated in over 20 published human clinical studies. In these studies, there were no reports of serious adverse effects or observed safety concerns. *B. coagulans* was generally well tolerated. The daily intake of *B. coagulans* was up to approximately  $20 \times 10^9$  cfu/day and the period of intervention ranged from a few days to approximately 13 weeks. In a few studies, mild to moderate gastrointestinal symptoms with intake of *B. coagulans* were reported. However, the effects were generally self-limiting and reversible. Overall, the findings from these studies in both healthy and compromised individuals did not reveal any evidence of pathogenicity or toxicity following ingestion of *B. coagulans*.

The safety of multiple strains of *B. coagulans* has been extensively examined in a variety of animal toxicity studies. Findings from acute toxicity, subchronic toxicity, chronic toxicity (one year) and reproductive toxicity studies suggest that *B. coagulans* is not toxic. The longest duration oral toxicity study of *B. coagulans* was a one-year repeat dose toxicity study of *B. coagulans* GBI-30, 6086. In the subchronic toxicity study with *B. coagulans* SANK70258 (mother strain of subject of the present GRAS), the NOAEL was determined to be 2000 mg/kg bw/day, equivalent to a daily intake of  $1.02 \times 10^{12}$  cfu/kg bw/day. Additionally, the lack of adverse effects during postnatal observations in the F1 generation part of the reproductive study is of additional significance to the consideration of use of *B. coagulans* in infant formula. The *B. coagulans* strains tested in these studies are very similar to the subject of the present GRAS assessment. The data from these studies can be used to support the safety of *B. coagulans* SNZ 1969 spore preparation.

The studies described in this GRAS notification, including those described in the previous GRAS notice by Sanzyme Ltd. (GRN 597) on GRAS assessment, *B. coagulans* SNZ 1969 spore preparation, and incorporated herein by reference and studies on infant populations, did not reveal any toxicological adverse effects of *B. coagulans* SNZ 1969 and other strains on infant growth that is considered as a primary determinant of safety for infant formulas or any other toxicologically relevant outcome measures. The available safety related studies did not reveal any target organs, including any systems of particular susceptibility in infants. Additionally, bioinformatics analysis from whole genome sequence have shown that *B. coagulans* SNZ 1969 is non-pathogenic, non-toxigenic, and does not harbor transferable antibiotic resistance genes.

It should be noted that during our review, and in gathering information related to *B. coagulans*, we cited a publication authored by Dr. Chandra (Chandra, 2002). This article was also cited in other GRAS Notices on *B. coagulans* submitted to FDA. Subsequently, we became aware of some issues associated with Dr. Chandra involving scientific fraud which has resulted in some of his publications being retracted from some scientific publications. However, his 2002 publication that is cited in this document has not been retracted at this time. The Expert Panel is aware of this issue and has elected not to include the information in this publication as part of its evaluation of this GRAS assessment. The Expert Panel's conclusion is based on the totality of the evidence absent this publication.

The safety conclusion of *B. coagulans* SNZ 1969 is based on the totality of the available evidence, including phenotypic and genotypic characterization, whole genome sequence and bioinformatics analysis, traditional and current uses, and animal and human studies, including those for other similar strains. The published data satisfy the common knowledge element of the GRAS standard and provide evidence that there is reasonable certainty that consumption of *B. coagulans* SNZ 1969 for its intended use in non-exempt term infant formula up to a maximum addition level of  $2 \times 10^8$  cfu/100 ml is safe.

The evidence of *B. coagulans* SNZ 1969 safety or lack of adverse effects is supported by:

- Use in the production of a traditional protein-rich food known as *ugba*.
- No pathogenic and toxicogenic effects noted.
- Transient nature of *B. coagulans* in the GI tract and consumption either in foods or as a dietary supplement does not have any cumulative effect that would affect its safety.
- No toxicity reported in animal studies at doses up to  $20 \times 10^9$  spores/kg bw/day.
- No adverse effects noted in several human studies, including studies of up to 1-year duration and in susceptible groups (infants/children).
- *B. coagulans* SNZ 1969 is marketed for over 40 years without any reports of significant adverse effects.

In summary, on the basis of scientific procedures<sup>9</sup>, including knowledge from a traditional and current exposure to *B. coagulans* SNZ 1969, the consumption of *B. coagulans* SNZ 1969 as an added food ingredient in infant formula at levels up to  $2 \times 10^8$  cfu per 100 ml infant formula as ready for consumption resulting in a maximum estimated daily intake of  $4.27 \times 10^8$  cfu *B. coagulans* SNZ 1969 spores/kg bw is considered safe. The intended uses are compatible with current regulations, *i.e.*, *B. coagulans* SNZ 1969 is used in infant formula (as described in this document) and is produced according to current good manufacturing practices (cGMP).

---

<sup>9</sup> 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.




#### 6.4. Expert Panel Conclusion

Based on a critical evaluation of the publicly available data, described and summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that *Bacillus coagulans* SNZ 1969 spore preparation, meeting the specifications cited above, and when used as a food ingredient in infant formula at use levels of  $2 \times 10^8$  cfu per 100 ml of formula, as ready for consumption, resulting in a maximum estimated daily intake of  $4.27 \times 10^8$  cfu *B. coagulans* SNZ 1969 spores/kg bw, is considered safe and Generally Recognized As Safe (GRAS).

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that *Bacillus coagulans* SNZ 1969 spore preparation, when used as described, is GRAS, based on scientific procedures.

#### Signatures



Robert L. Martin, Ph.D.

May 9, 2019

Date



Douglas L. Archer, Ph.D.

May 11, 2019

Date



Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.  
**Advisor to Expert Panel**

May 15, 2019

Date

## 7. Part VII – SUPPORTING DATA AND INFORMATION

- Adams, M.R., 1999. Safety of industrial lactic acid bacteria. *J. Biotechnol.* 68, 171-178.
- Akagawa Y, Ohnishi Y, Takaya M, Watanabe Y., 2015 Safety assessment of probiotic bacteria, *Bacillus coagulans* strain SANK70258, in rats. *Fundam. Toxicol. Sci.* 3(6):243-250.
- Anonymous, 2002. *Lactobacillus sporogenes*. *Altern. Med. Rev.* 7, 340-342.
- Ara, K., Meguro, S., Hase, T., Tokimitsu, L., Otsuji, K., Kawai, S., Ito, S., Iino, H., 2002. Effect of spore-bearing lactic acid-forming bacteria (*Bacillus coagulans* SANK 70258) administration on the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics in humans and rats. *Microbial Ecol. Health Disease* 14,4-13.
- Asemi, Z., Alizadeh, S.A., Ahmad, K., Goli, M., Esmailzadeh, A., 2015. Effects of beta-carotene fortified synbiotic food on metabolic control of patients with type 2 diabetes mellitus: A double-blind randomized cross-over controlled clinical trial. *Clin. Nutr.* 35(4):819-825.
- Asgarshirazi, M., Shariat, M., Dalili, H., 2015. Comparison of the Effects of pH-Dependent Peppermint Oil and Synbiotic Lactol (*Bacillus coagulans* + Fructooligosaccharides) on Childhood Functional Abdominal Pain: A Randomized Placebo-Controlled Study. *Iran Red Crescent Med J.* 17(4):e23844.
- Ashtekar, A., 2012. Probiotics evaluation studies on *B. coagulans*. Shubhda Research Institute. Study report provided by Sanzyme Biologics.
- Astegiano, M., Pellicano, R., Terzi, E., Simondi, D., Rizzetto, M., 2006. Treatment of irritable bowel syndrome. A case control experience. *Minerva Gastroenterol. Dietol.* 52, 359-363 (information based on abstract).
- Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008) The RAST Server: Rapid Annotations using Subsystems Technology, *BMC Genomics* 9, 75.
- Bahmani F, Tajadadi-Ebrahimi M, Kolahdooz F, Mazouchi M, Hadaegh H, Jamal AS, Mazroii N, Asemi S, Asemi Z. 2016. The Consumption of Synbiotic Bread Containing *Lactobacillus sporogenes* and Inulin Affects Nitric Oxide and Malondialdehyde in Patients with Type 2 Diabetes Mellitus: Randomized, Double-Blind, Placebo-Controlled Trial. *J. Am. Coll. Nutr.* 35(6):506-513.
- Banerjee, C., Bustamante, C.I., Wharton, R., Talley, E., Wade, J.C., 1988. *Bacillus* infections in patients with cancer. *Arch. Intern. Med.* 148, 1769-1774.
- Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J., and Henriques, A.O., 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology* 71:968-978.
- Baron M., 2009. A patented strain of *Bacillus coagulans* increased immune response to viral challenge. *Postgrad Med.* 121(2):114-118.

- Bergey, D.H., Breed, R.S., Murray, E.G.D., Parket-Hitchens, A., 1939. Bergey's manual of determinative bacteriology. 5th ed. Baltimore: William & Wilkins Co.
- Butte, N.F., Fox, M.K., Briefel, R.R., Siega-Riz, A.M., Dwyer, J.T., Deming, D.M., Reidy, K.C., 2010. Nutrient intakes of US infants, toddlers, and preschoolers meet or exceed dietary reference intakes. *J. Am. Diet. Assoc.* 110(12 Suppl):S27-37.
- Catanzaro, J.A., Green, L., 1997. Microbial ecology and probiotics in human. *Alter. Med. Rev.* 2, 296-305.
- Cavazzoni, V., Adami, A., Castrovilli, C., 1998. Performance of broiler chickens supplemented with *Bacillus coagulans* as probiotic. *British Poultry Sci.* 39, 526-529.
- Chandra, R.K., 2002. Effect of Lactobacillus on the incidence and severity of acute rotavirus diarrhoea in infants. A prospective placebo-controlled double-blind study. *Nutr. Res.* 22, 65:69.
- Cui, Y., Wan, F. C., Tang, D.L., Wu, S.H., 2004. Efficacy of *Bacillus coagulans* tablets in the treatment of acute and chronic diarrhea. *Int. J. Immunotherapy* 20, 17-22.
- Deodhar, J., 1984. Trial of Sporlac in neonatal diarrhea. *Maharashtra Medical Journal* 30(10): 235-236.
- Dhongade, R.K., Anjanyulu, R., 1977. Sporlac studies. Abstract from Maharashtra Medical Journal XXITI 473-474.
- Dolin, B.J., 2009. Effects of a proprietary *Bacillus coagulans* preparation on symptoms of diarrhea-predominant irritable bowel syndrome. *Methods Find Exp. Clin. Pharmacol.* 31(10):655-659.
- Doron, S., Gorbach, S.L., 2006. Probiotics: their role in the treatment and prevention of disease. *Expert. Rev. Anti. Infect. Ther.* 4, 261-275.
- Doron, S.I., Hibberd, P.L., Gorbach, S.L., 2008. Probiotics for prevention of antibiotic associated diarrhea. *J. Clin. Gastroenterol.* 42 (Suppl 2), S58-63.
- DSHEA, 1994. Dietary Supplements Health and Education Act of 1994. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC.
- Dutta, P., Mitra, U., Dutta, S., Rajendran, K., Saha, T.K., Chatterjee, M.K., 2011. Randomised controlled clinical trial of *Lactobacillus sporogenes* (*Bacillus coagulans*), used as probiotic in clinical practice, on acute watery diarrhoea in children. *Trop Med Int Health.* 16:555-561.
- EFSA, 2012a. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J* 10 (6):2740.
- EFSA, 2012. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). *EFSA Journal* 10(12):1-84.
- El Ghachi, M. El., Bouhss, A., Blanot, D., Mengin-Lecreulx, D., 2004. The bacA gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. *J. Biol. Chem.* 279(29):30106-30113.

- Endres, J.R., Clewell, A., Jade, K.A., Farber, T., Hauswirth, J., Schauss, A.G., 2009. Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem Toxicol.* 47:1231-1238.
- Endres, J.R., Qureshi, L., Farber, T., Hauswirth, J., Hirka, G., Pasics, L., Schauss, A.G., 2011. One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem Toxicol.* 49:1174-1182.
- Faith, N.G., Kim, J.W., Azizoglu, R., Kathariou, S., Czuprynski, C., 2012. Purine biosynthesis mutants (purA and purB) of serotype 4b *Listeria monocytogenes* are severely attenuated for systemic infection in intragastrically inoculated A/J Mice. *Foodborne Pathog. Dis.* 9:480-486.
- FDA, 2001. Partial List of Enzyme Preparations That Are Used In Foods. Food and Drug Administration. Available at: <http://www.fda.gov/Food/FoodIngredientsPackaging/ucrn084292.htm>
- FDA, 2012. Preparation of *Bacillus coagulans* strain GBI-30, 6086 spores. Agency Response Letter GRAS Notice No. GRN 000399 and additional information. Available at: <http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=399>
- Fooks, L.J., Gibson, G.R., 2002. Probiotics as modulators of the gut flora. *Br. J. Nutr.* 88 (Suppl 1), S39-49.
- Fomon, S., 1974. Voluntary food intake and its regulation. In: *Infant Nutrition, Second Edition.* Philadelphia: W.B. Saunders Company: 20-33.
- Ganeden, 2016. GRN 660. *Bacillus coagulans* GBI-30, 6086 (Complete Notice and Agency Response Letter). GRAS Notice Inventory.
- Ganeden, 2011. Notice to US Food and Drug Administration that *Bacillus coagulans* GBI-30, 6086, a Novel Probiotic, is Generally Recognized as Safe for use in Foods. Ganeden Biotech, Inc. Available at: <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=39>.
- Grimes, C.A., Szymlek-Gay, E.A., et al., 2015. Food Sources of Total Energy and Nutrients among U.S. Infants and Toddlers: National Health and Nutrition Examination Survey 2005-2012. *Nutrients.* 7(8):6797-836.
- Gu, S.B., Zhao, L.N., Wu, Y., Li, S.C., Sun, J.R., Huang, J.F., Li, D.D., 2015. Potential probiotic attributes of a new strain of *Bacillus coagulans* CGMCC 9951 isolated from healthy piglet feces. *World J Microbial Biotechnol.* 31(6):851-863.
- Guida, V.O., Zuccas, A.W., Guida, H.A., 1958. Studies on the stimulating action of suspensions of *Bacillus coagulans*, *Bacillus polymyxa* & *Bacillus subtilis* administered orally for deficiency of normal children from 3 to 5 years of age. *Rev. Bras. Med.* 15, 481-484. (Article in Portuguese).
- Guida, V.O., Guida, H.A., 1959. Treatment of iron-deficiency hypochromic anemia with *Bacillus cereus* & *Bacillus coagulans* in patients with enterocolitis. *Rev. Bras. Gastroenterol.* 11, 17-22. (Article in Portuguese).

- Heikkinen, J. 2017. Genome Sequencing, Identification and Bioinformatic Safety Assessment of *Bacillus coagulans*. Biosafe- Biological Safety Solutions Ltd., Finland. Report No.: T242R324/2017. pp. 1-10. Unpublished report provided by Sanzyme Biologics.
- Heikkinen, J. 2017a. Characterization of Antibiotic Susceptibility of *Bacillus coagulans* SNZ 1969. Biosafe- Biological Safety Solutions Ltd., Finland. Study Report No.: T242R315/2017. pp. 1-4. Unpublished report provided by Sanzyme Biologics.
- Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Inter. J. Food Microbiol* 24, 343-362.
- Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci, M.C. and Cutting, S.M., 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol.* 105:510-520.
- Horowitz-Wlassowa, L.M., Nowotelnow, N.W., 1932. Uber eine sporogenes Milchsäurebakterienart, *Lactobacillus sporogenes* n. sp. *Zentralb. F. Bakt. II Abt.* 87, 331.
- Hun, L., 2009. *Bacillus coagulans* significantly improved abdominal pain and bloating in patients with IBS. *Postgrad Med.* 121:119-124.
- Iino, H., Matsumoto, Y., Ito, M., Abe, S., Sugara, T., 1997a. Research on the fecal improvement effect due to lactic acid forming bacillus bacteria (*Bacillus coagulans*). *Prog. Med.* 17: 3299-3302. (Article in Japanese)
- Iino, H., Matsumoto, Y., Ito, M., Abe, S., Sugara, T., 1997b. Research on the human intestinal flora improvement effect by lactic acid forming bacillus bacteria (*Bacillus coagulans*). *Prog. Med.* 17: 3303-3308. (Article in Japanese)
- Johnston, B.C., Supina, A.L., Ospina, M., Vohra, S., 2007. Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *Cochrane Database Syst. Rev.* 18, (2):CD004827.
- Kajimoto, O., Suwa, M., Takahashi, Y., Uchida, N., Hayama, M., Tamura, M., Nakagawa, S., Kajimoto, Y., 2005. Effects of *Bacillus coagulans* SANK70258 on seasonal allergic rhinitis (Japanese Cedar Pollinosis): A randomized, placebo controlled, double-blind study. *J. New Rem. Clin.* 54(11): 107-125. (Article in Japanese)
- Kalman, D.S., Schwartz, H.I., Alvarez, P., Feldman, S., Pezzullo, J.C., Krieger, D.R., 2009. A prospective, randomized, double-blind, placebo-controlled parallel-group dual site trial to evaluate the effects of a *Bacillus coagulans*-based product on functional intestinal gas symptoms. *BMC Gastroenterol.* 9:85.
- Khatri, I., Sharma, S., Ramya, T.N., Subramanian, S., 2016. Complete Genomes of *Bacillus coagulans* S-lac and *Bacillus subtilis* TO-A JPC, Two Phylogenetically Distinct Probiotics. *PLoS One.* 11(6):e0156745.
- Kimmel M, Keller D, Farmer S, Wanino DE. 2010. A controlled clinical trial to evaluate the effect of Ganeden BC(30) on immunological markers. *Methods Find Exp Clin Pharmacol.* 32(2): 129-132.
- Konuray, G., Erginkaya, Z., 2018. Potential use of *Bacillus coagulans* in the food industry. *Foods (Basel, Switzerland)* 7(6):92. doi:10.3390/foods7060092

- Kumar, B. S., 2018. Acute Oral Toxicity Study of *Bacillus Coagulans* in Wistar Rats. Study Number PCT/0218/11. Study Report provided by Sanzyme Biologics.
- Labalestra, V., Pannone, V., Iacono, O., Federici, T., Maiella, G., Oliva, S., Tricarico, A., Paganelli, M., Cucchiara, S., 2008. Effect of a combination of symethicone and *Bacillus coagulans* (Colinox) on the gastric emptying time in infants with symptomatic gastroesophageal reflux (GER): A randomized, double-blind, placebo-controlled, cross-over study. *Dige. Liver Dis.* 40, A 72 (abstract).
- La Rosa, M., Bottaro, G., Gulino, N., Gambuzza, F., Di Forti, F., Ini, G., Tornambe, E., 2003. Prevention of antibiotic-associated diarrhea with *Lactobacillus sporogens* and fructooligosaccharides in children. A multicentric double-blind vs placebo study. *Minerva Pediatr.* 55, 447-452.
- Lavanya, S., 2015. 28 Day Repeated Dose Oral Toxicity Study of *Bacillus coagulans* (SNZ - 1969) in Wistar rats. Study No. : 13\_00\_004. Study Report provided by Sanzyme.
- Lee, Y.-K., Salminen, S., 1995. The coming age of probiotics. *Trends in Food Sci. Technol.* 6, 241-245.
- Losada, M.A., Olleros, T., 2002. Towards a healthier diet for the colon: the influence of fructooligosaccharides and lactobacilli on intestinal health. *Nutr. Res.* 22, 71-84.
- Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., Karri, S.K., 2016. *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study. *Nutr J.* 15:21.
- Majeed, M., Prakash, L., 1998. *Lactospore®: The Effective Probiotic*. Piscataway, NJ: NutriScience Publishers, Inc. (ISBN: 0-964785-4-1); 1998. pp. 1-56.
- Mandel, D.R., Eichas, K., Holmes, J., 2010. *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Complement Altern Med.* 10:1.
- McGillivray SM, Ebrahimi CM, Fisher N, Sabet M, Zhang DX, Chen Y, Haste NM, Aroian RV, Gallo RL, Guiney DG, Friedlander AM, Koehler TM, Nizet V., 2009. ClpX contributes to innate defense peptide resistance and virulence phenotypes of *Bacillus anthracis*. *J Innate Immun.* 1(5):494-506.
- Miele, A., Goldstein, B.P., Bandera, M., Jarvis, C., Resconi, A., Williams, R.J., (1994). Differential susceptibilities of enterococcal species to elfamycin antibiotics. *Journal of Clinical Microbiology* 32(8): 2016-2018.
- Mohan, J.C., Arora, R., Khalilullah, M., 1990a. Preliminary observations on effect of *Lactobacillus sporogenes* on serum lipid levels in hypercholesterolemic patients. *Indian J. Med. Res.* 92, 431-432.
- Mohan, J.C., Arora, R., Khalilullah, M., 1990b. Short term hypolipidemic effects of oral *Lactobacillus sporogenes* therapy in patients with primary dyslipidemias. *Indian Heart J.* 42, 361-364.

- Moldenhauer, J.E., Bass, S., Rubio, S.L., 1996. Antimicrobial resistance of three species of *Bacillus* to thirty various antimicrobial agents. *PDA J. Pharm. Sci. Technol.* 50, 324-325.
- Nyangale EP, FarmerS, Cash HA, Keller D, ChernoffD, Gibson GR., 2015. *Bacillus coagulans* GBI30, 6086 Modulates *Faecalibacterium prausnitzii* in Older Men and Women. *J Nutr.* 145(7): 1446-1452.
- ODS/NLM. (2019). *Bacillus coagulans*. In Dietary Supplement Label Database. National Institutes of Health Office of Dietary Supplements (ODS) and the US National Library of Medicine. Available online at: <https://dslid.nlm.nih.gov/dslid/rptQSearch.jsp?item=Bacillus+Coagulans&db=adslid>
- Okonko, I.O., Olabode, O.P., Okeleji, O.S., 2006. The role of biotechnology in the socio-economic advancement and national development: An Overview. *African J. Biotechnol.* 5, 2354-2366.
- Onofiok, N., Nnanyelugo, D.O., Ukwondi, B.E., 1996. Usage patterns and contribution of fermented foods to the nutrient intakes of low income households in Emene, Nigeria. *Plant Foods Human Nutr.* 49, 199-211.
- Ouwehand, A.C., Salminen, S., Roberts, P.J., Ovaska, J., Salminen, E., 2003. Disease-dependent adhesion of lactic acid bacteria to the human intestinal mucosa. *Clin. Diagn. Lab. Immunol.* 10, 643-646.
- Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H. Y., Cohoon, M., de Crecy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E. D., Gerdes, S., Glass, E. M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A. C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., Pusch, G. D., Rodionov, D. A., Ruckert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O., and Vonstein, V.. 2005. The Subsystems Approach to Genome Annotation and its Use in the Project to Annotate 1000 Genomes. *Nucleic Acids Res* 33, 5691-5702.
- Rangarajan, N., Patel, B., Rangarajan, P., Marczely, J., Dheer, R., Chordia, T., Dunn, S.R., Friedman, E.A., 2005. Probiotic amelioration of azotemia in 5/6th nephrectomized SpragueDawley rats. *TheScientificWorld J.* 5, 652-660.
- Rogha, M., Esfahani, M.Z., Zargarzadeh, A.H., 2014. The efficacy of asynbiotic containing *Bacillus coagulans* in treatment of irritable bowel syndrome: arandomized placebo-controlled trial. *Gastroenterol. Hepatol. Bed Bench.* 7(3):156-163.
- Salminen, S., Isolauri, E., Salminen, E., 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie Van Leeuwenhoek* 70, 347-358.
- Sarles, W.B., Hammer, B.W., 1932. Observations on *Bacillus coagulans*. *J. Bacterial.* 23(4):301-314.
- Sanders, N.E., Morelli, L., Bush, S., 2001. "*Lactobacillus sporogenes*" is not a *Lactobacillus* probiotic. *ASM News* 8, 6.
- Sanders, N.E., Morelli, L., Tompkins, T.A., 2003. Sporeformers as human probiotics: *Bacillus*, *Sporolattobacillus*, and *Brevibacillus*. *Comp. Rev. Food Sci. Food Safety* 2, 101-110.

- Sankyo Co. Ltd., Tokyo, Japan, 1968. Outline of Lactis for animal use-A preparation of sporebearing lactic-acid forming *Bacillus* for veterinary use, 1-16.
- Sari, F.N., Dizdar, E.A., Oguz, S., Erdeve, O., Uras, N., Dilmen, U., 2011. Oral probiotics: *Lactobacillus sporogenes* for prevention of necrotizing enterocolitis in very low-birth weight infants: a randomized, controlled trial. *Eur. J. Clin. Nutr.* 65(4):434-439.
- Schauer, K., Geginat, G., Liang, C., Goebel, W., Dandekar, T., Fuchs, T. M., 2010. Deciphering the intracellular metabolism of *Listeria monocytogenes* by mutant screening and modelling. *BMC genomics*, 11(1):573.
- Shakeri H, Hadaegh H, Abedi F, Tajabadi-Ebrahimi M, Mazroii N, Ghandi Y, Asemi Z., 2014. Consumption of synbiotic bread decreases triacylglycerol and VLDL levels while increasing HDL levels in serum from patients with type-2 diabetes. *Lipids* 49(7):695-670.
- Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (eds). 1986. *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams and Wilkins, Baltimore, MD.
- Soomro, A.H., Masud, T., Anwaar, K., 2002. Role of lactic acid bacteria (LAB) in food preservation and human health-A review. *Pakistan J. Nutr.* 1, 20-24.
- Sorokulova I.B., Pinchuk I.V., Denayrolles M., Osipova I.G., Huang J.M., Cutting S.M., Urdaci M.C., 2008. The safety of two *Bacillus* probiotic strains for human use. *Digestive Diseases and Sciences* 53: 954-963.
- Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., and Oggioni, M.R., 2000. On the fate of ingested *Bacillus* spores. *Research in Microbiology* 151:361-368.
- Sudha RM, Bhonagiri S., 2012b. Efficacy of *Bacillus coagulans* strain IS-2 in the treatment of patients with acute diarrhea. *International Journal of Probiotics and Prebiotics.* 7(1):33-37.
- Sudha RM, Radkar N, Maurya R. 2011b. Effect of supplementation of *Bacillus coagulans* Unique IS-2 (ATCC PTA-11748) on hypercholesterolemic subjects: a clinical study. *Inter J Probiotics Prebiotics.* 6(2):89-94.
- Sudha, R.M., Sunita, M., Sekhar, B.M., 2011a. Safety studies of *Bacillus coagulans* Unique IS-2 in rats: Morphological, biochemical and clinical evaluations. *Int. J. Probiotics Prebiotics* 6:43-48.
- Sudha RM, Yelikar KA, Deshpande S. 2012a. Clinical Study of *Bacillus coagulans* Unique IS-2 (ATCC PTA-11748) in the Treatment of Patients with Bacterial Vaginosis. *Indian J Microbiol.* 52(3):396-399.
- Swamy, M.V., Soman, J.L., 2013. *Bacillus coagulans* strain SNZ1969 16S ribosomal RNA gene, partial sequence. Available at: <http://www.ncbi.nlm.nih.gov/nucleotide/KC146407>
- Tajadadi-Ebrahimi M, Bahmani F, Shakeri H, Hadaegh H, Hijijafari M, Abedi F, Asemi Z., 2014. Effects of daily consumption of synbiotic bread on insulin metabolism and serum high-sensitivity C-reactive protein among diabetic patients: a double-blind, randomized, controlled clinical trial. *Ann Nutr Metab.* 65(1):34-41.
- Tuohy, K.M., Pinart-Gilberga, M., Jones, M., Hoyles, L., McCartney, A.L., and Gibson, G.R., 2007. Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of



- healthy human volunteers and its impact on the faecal microflora. *Journal of Applied Microbiology* 102:1026-1032.
- Van Schaik, W., Château, A., Dillies, M. A., Coppée, J.Y., Sonenshein, A. L., Fouet, A. 2009. The global regulator CodY regulates toxin gene expression in *Bacillus anthracis* and is required for full virulence. *Infection and Immunity* 77(10):4437-4445.
- Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., Conrad, N., Dietrich, E. M., Disz, T., Gabbard, J. L., Gerder, S., Henry, C. S., Kenyon, R. W., Machi, D., Mao, C., Nordberg, E. K., Olsen, G. J., Murphy-Olson, D. E., Olson, R., Overbeek, R., Parrello, B., Pusch, G. D., Maulik, S., Vonstein, V., Warren, F. X., Yoo, H., Gerdes, S., 2017. Improvements to PATRIC, the all bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Research* 45(D1): D535-D542.
- Yang, O.O., Kelesidis, T., Cordova, R., Khanlou, H., 2014. Immunomodulation of antiretroviral drug-suppressed chronic HIV -1 infection in an oral probiotic double-blind placebo-controlled trial. *AIDS Res Hum Retroviruses*. 30(10):988-95.

## APPENDIX I

### 16S rRNA profile of *Bacillus coagulans* SNZ 1969

LOCUS KC146407 1491 bp DNA linear BCT 31-OCT-2013  
DEFINITION *Bacillus coagulans* strain SNZ 1969 16S ribosomal RNA gene, partial sequence.  
ACCESSION [KC146407](#)  
VERSION KC146407.1  
KEYWORDS  
SOURCE *Bacillus coagulans*  
ORGANISM [Bacillus coagulans](#)  
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.  
REFERENCE 1 (bases 1 to 1491)  
AUTHORS Swamy,M.V. and Soman,J.L.  
TITLE Screening and development of *Bacillus coagulans* for various probiotic applications  
JOURNAL Unpublished  
REFERENCE 2 (bases 1 to 1491)  
AUTHORS Swamy,M.V. and Soman,J.L.  
TITLE Direct Submission  
JOURNAL Submitted (03-NOV-2012) Research and Development Division, Sanzyme Ltd, Gagan pahad, Shamshabad, Hyderabad, Andhrapradesh 501323, India  
COMMENT ##Assembly-Data-START##  
Sequencing Technology :: Sanger dideoxy sequencing  
##Assembly-Data-END##  
FEATURES  
source Location/Qualifiers  
1..1491  
organism="[Bacillus coagulans](#)"  
mol\_type="genomic DNA"  
strain="SNZ 1969"  
isolation\_source="green malt"  
db\_xref="[taxon:1398](#)"  
country="India"  
rRNA <1..>1491  
product="16S ribosomal RNA"  
BASE COUNT 363 a 366 c 474 g 287 t  
ORIGIN  
1 aggacgaacg ctggcggcgt gcctaataca tgcaagtctg gcgaccttt taaaagcttg  
61 cttttaaag gttagcggcg gacgggtgag taacacgtgg gcaacctgcc tgaagatcg  
121 ggataacgcc gggaaaccgg ggctaatacc ggatagttt ttctccgca tggaggaaaa  
181 aggaaagacg gcttcggctg tcacttacag atgggcccg cgcgcattag ctagtggtg  
241 gggtaacggc tcaccaaggc aacgatcgt agccgacctg agagggtgat cggccacatt  
301 gggactgaga cacggcccaa actcctacgg gaggcagcag tagggaatct tccgcaatgg  
361 acgaaagtct gacggagcaa gcgccgctga gtgaagaagg ccttcgggtc gtaaactct  
421 gttgccgggg aagaacaagt gccgtcga cagggcggcg ccttgacggt acccgccag  
481 aaagccaagg ctaactacgt gccagcagcc gcgtaatac gtaggtggca agcgtgtcc  
541 ggaattatgg kgggcgtaaa gcgcgcgag gcggtctctt aagtctgatg tgaatcttg  
601 cggctcaacc gcaagcggtc attggaaact gggaggcttg agtcagaag aggagagtgg  
661 aattccacgt gtagcgggta aatgcgtaga gatgtggagg aacaccagtg gcgaaggcgg  
721 ctctctggt tctgtaacct gcaacgctga ggccgaaag cgtggggagc aacaggatt  
781 agataccctg gtagtccacg ccgtaaacga tgagtctaa gtgttagagg gtttccgcc  
841 tttagtctg cagctaacgc attaacact ccgctgggg agtacggccg caaggctgaa

901 actcaaagga attgacgggg gcccgacaaa gcggtggagc atgtggttta attcgaagca  
961 acgcaagaa ccttaccagg tcttgacalc ctctgacclc cctggagaca gggccttccc  
1021 ctlcggggga cagagtgaca ggtgggcat ggtgtcgtc agctcgtgc gtgagatgt  
1081 gggtaagtc ccgcaacgag cgcaaccctt gacctagt gccagcaltc agtggggcac  
1141 tctaaggtga ctgccggtga caaacggag gaaggtgggg atgacgtcaa atcatcatgc  
1201 cccttatgac ctgggtaca cacgtgtac aatggatgtt acaaagggt gcgagaccgc  
1261 gaggtaagc caatcccaga aaaccattcc cagttcggat tgcaggctgc aaccgcctg  
1321 catgaagccg gaatcgctag taatcgcgga tcaccatgcc gcgggtgaat acgtcccgg  
1381 gccttgata caccgcccgt cacaccacga gagttgtaa caccgaagt cggtaggta  
1441 accttacgg agccagccgc cgaaggtggg acagatgatt ggggtgatct a

//

## APPENDIX II

Analytical data from five manufacturing lots

### Specifications of *B. coagulans* SNZ 1969 spore preparation from five non-consecutive manufacturing lots (Sanzyme Biologics, 2018)

Test	Standard specifications	Batch Numbers				
Appearance	Dark grayish white powder	Complies	Complies	Complies	Complies	Complies
Identification	A. Aerobic, gram positive thermostable spores identified by blue colored rods and red colored spores as seen under microscope, when stained by basic fuchsin solution B. Producing Lactic Acid	Complies	Complies	Complies	Complies	Complies
Loss on drying (105° for 1 hour)	NMT 5% w/w	3.96%w/w	3.93%w/w	3.96%w/w	3.98%w/w	4.05%w/w
Lactic acid producing capacity	Not Less Than 10 ml of 0.05 M NaOH is consumed	14.40ml	15.00ml	15.10ml	14.80ml	14.40ml
Viable spore	NLT 10 x 10 <sup>10</sup> cfu/g	118.50 Billion cfu/g	116.92 Billion cfu/g	117.42 Billion cfu/g	113.96 Billion cfu/g	114.84 Billion cfu/g
Heat Resistant Ratio (At 85°C)	Not Less Than 70%	81.95%	81.72%	82.12%	82.33%	81.73%
<b>Heavy Metals</b>						
Arsenic	NMT 1 ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm
Lead	NMT 1 ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm
Mercury	NMT 1 ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm
Cadmium	NMT 1 ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm	<1.0ppm
<b>Total Viable Aerobic Counts</b>						
Total bacterial counts (other organisms)	NMT 0.1 million cfu/g	3500cfu/g	7000cfu/g	8000cfu/g	7500cfu/g	7000cfu/g
Yeast and Mold	NMT 100 cfu/g	5cfu/g	5cfu/g	5cfu/g	5cfu/g	5cfu/g
<i>Escherichia coli</i>	Negative/10 g	Absent	Absent	Absent	Absent	Absent
<i>Salmonella</i>	Negative/10 g	Absent	Absent	Absent	Absent	Absent
<i>Pseudomonas aeruginosa</i>	Negative/1 g	Absent	Absent	Absent	Absent	Absent
<i>Staphylococcus aureus</i>	Negative/1 g	Absent	Absent	Absent	Absent	Absent

<i>Bacillus cereus</i>	Less than 10cfu/g	<10cfu/g	<10cfu/g	<10cfu/g	<10cfu/g	<10cfu/g
<i>Lysteria monocytogens</i>	Negative/25 g	Absent	Absent	Absent	Absent	Absent
<i>Cronobacter sakazakii</i>	Negative/100g	Complies	Complies	Complies	Complies	Complies

**From:** [Madhu Soni](#)  
**To:** [Anderson, Ellen](#)  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation  
**Date:** Friday, January 17, 2020 10:22:02 AM  
**Attachments:** [image001.png](#)  
[image002.png](#)  
[image003.png](#)

---

Dear Dr. Anderson,

I have confirmed with our client and it is our oversight. You are correct that the BAM chapter for *Cronobacter* is Chapter 29. Sorry for the oversight .

We look forward to the finalization of this GRAS notice.

Best regards

Madhu

-----  
**Madhu G. Soni, PhD, FACN, FATS**

Soni & Associates Inc.

749 46<sup>th</sup> Square

Vero Beach, FL 32968, USA

Phone: +1-772-299-0746; Cell: +1-772-538-0104

[www.soniassociates.net](http://www.soniassociates.net)

---

**From:** Anderson, Ellen [mailto:[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)]  
**Sent:** Wednesday, January 15, 2020 2:46 PM  
**To:** 'Madhu Soni' <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Subject:** FW: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Hello Dr. Soni,

We have one quick follow-up question regarding the specified methodology for *Cronobacter sakazakii* in the attached spreadsheet. We believe there is a typo in the BAM chapter listed in the Method column. The BAM chapter for *Cronobacter* is [Chapter 29](#), not Chapter 25. Please confirm the correct chapter number.

Thank you,

Ellen

**Ellen Anderson**

Consumer Safety Officer

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

Tel: 240-402-1309

[ellen.anderson@fda.hhs.gov](mailto:ellen.anderson@fda.hhs.gov)



---

**From:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>

**From:** [Madhu Soni](#)  
**To:** [Anderson, Ellen](#)  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation  
**Date:** Thursday, December 19, 2019 1:30:40 PM  
**Attachments:** [image001.png](#)  
[image002.png](#)  
[image003.png](#)  
[Specs.xls](#)

---

Dear Dr. Anderson,

Sorry I forgot to attach another file (Excel sheet- please see attached) received from the client. Please accept my apology for the oversight.

Best regards

Madhu

---

**From:** Anderson, Ellen [mailto:[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)]  
**Sent:** Thursday, December 19, 2019 8:56 AM  
**To:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Thank you, Dr. Soni. I will pass this along to our reviewers and get back to you if we have any follow-up questions.

Sincerely,  
Ellen

---

**From:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Sent:** Thursday, December 19, 2019 8:37 AM  
**To:** Anderson, Ellen <[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Dear Dr Anderson,

I am not sure whether the attached revised letter, just received from my client, replacing IH protocol with In-House protocol will be satisfactory for your purposes. Please let me know. Thanks

Best regards

Madhu

---

**From:** Madhu Soni [<mailto:sonim@bellsouth.net>]  
**Sent:** Wednesday, December 18, 2019 3:18 PM  
**To:** 'Anderson, Ellen' <[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Dear Dr Anderson,

Sorry for my oversight and should have checked your email carefully. If I understand correctly, you need the analytical methods used in the specification parameters. I have requested the client to provide the methods used for the specification parameters. It appears

that during preparation of this GRAS notice, I also missed this point. Again my apology for the oversight.

Best regards

Madhu

---

**From:** Anderson, Ellen [<mailto:Ellen.Anderson@fda.hhs.gov>]  
**Sent:** Wednesday, December 18, 2019 1:28 PM  
**To:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Subject:** FW: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Hello Dr. Soni,

Thank you for your email and attached statement. The statement your forwarded says, "We confirm that the analytical test method for determining the potency of "Bacillus coagulans SNZ 1969" has been validated as per IH protocol."

Could you please elaborate on what is meant by "IH protocol"?

Thank you,  
Ellen

**Ellen Anderson**

*Consumer Safety Officer*

**Center for Food Safety and Applied Nutrition**  
**Office of Food Additive Safety**  
**U.S. Food and Drug Administration**  
Tel: 240-402-1309  
[ellen.anderson@fda.hhs.gov](mailto:ellen.anderson@fda.hhs.gov)



---

**From:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Sent:** Wednesday, December 18, 2019 10:59 AM  
**To:** Anderson, Ellen <[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Dear Dr Anderson,

Thank you for your email and the update.

Please find attached, an official statement from Sanzyme as regards validation of analytical methods used. If you have any questions, please let me know.

Best regards

Madhu

.....  
Madhu Soni, PhD, FACN, FATS  
Soni & Associates Inc.

th



749 46 Square  
Vero Beach, FL 32968, USA  
Phone: [+1-772-299-0746](tel:+1-772-299-0746)  
Cell: [+1-772-538-0104](tel:+1-772-538-0104)  
[www.soniassociates.net](http://www.soniassociates.net)

---

**From:** Anderson, Ellen [<mailto:Ellen.Anderson@fda.hhs.gov>]  
**Sent:** Monday, December 16, 2019 10:44 AM  
**To:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Good morning Dr. Soni,

We are finishing up our review of GRN 864 and have one more item to clarify. Please provide a statement that all analytical methods used to analyze the batches of *B. coagulans* SNZ 1969 spore preparation for conformance with the stated specifications have been validated for that particular purpose.

Thank you,  
Ellen  
**Ellen Anderson**  
*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
Tel: 240-402-1309  
[ellen.anderson@fda.hhs.gov](mailto:ellen.anderson@fda.hhs.gov)



---

**From:** Madhu Soni <[sonim@bellsouth.net](mailto:sonim@bellsouth.net)>  
**Sent:** Wednesday, October 02, 2019 10:35 AM  
**To:** Anderson, Ellen <[Ellen.Anderson@fda.hhs.gov](mailto:Ellen.Anderson@fda.hhs.gov)>  
**Subject:** RE: GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Dear Dr. Anderson,  
I have received responses to your queries on GRN 864 from our client (Sanzyme). Please see below clarifications to your two queries:

**FDA Query 1.** Please state the intended protein source/sources (milk, soy, whey, etc.) of infant formula to which the ingredient would be added.

**Response:** The intended protein sources of infant formula to which *Bacillus coagulans* SNZ 1969 will be added are Whey, Casein, Milk and Soy. In this regard, please note that Sanzyme will market *B. coagulans* SNZ 1969 to the manufacturer of Infant Formula, who will be marketing the infant formula as per US regulations.

**FDA Query 2.** Please describe the source of maltodextrin that is used in the spore preparation.

**Response:** The maltodextrin used in the spore preparation is from sources such as corn,

potatoes or rice (21 CFR 184.1444).

Hope the above responses addresses your queries. If you have any questions, please let us know.

Best regards

Madhu

-----  
Madhu Soni, PhD, FACN, FATS  
Soni & Associates Inc.  
749 46<sup>th</sup> Square  
Vero Beach, FL 32968, USA  
Phone: [+1-772-299-0746](tel:+1-772-299-0746)  
Cell: [+1-772-538-0104](tel:+1-772-538-0104)  
[www.soniassociates.net](http://www.soniassociates.net)

---

**From:** Anderson, Ellen [<mailto:Ellen.Anderson@fda.hhs.gov>]

**Sent:** Tuesday, September 17, 2019 1:23 PM

**To:** [sonim@bellsouth.net](mailto:sonim@bellsouth.net)

**Subject:** GRN 864 - Bacillus coagulans SNZ 1969 spores preparation

Dear Dr. Soni,

As part of our ongoing evaluation of GRN 864 *Bacillus coagulans* SNZ 1969 spores preparation, we noted the following questions that require clarification.

1. Please state the intended protein source/sources (milk, soy, whey, etc.) of infant formula to which the ingredient would be added.
2. Please describe the source of maltodextrin that is used in the spore preparation.

We respectfully request a response by October 3, 2019. Please contact me if you have any questions.

Sincerely,

Ellen

**Ellen Anderson**

*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
Tel: 240-402-1309  
[ellen.anderson@fda.hhs.gov](mailto:ellen.anderson@fda.hhs.gov)



Date: 17/12/2019

**TO WHOM SO EVER IT MAY CONCERN**

We confirm that the Analytical Test Method for determining Appearance, Identity & Assay/ Potency of "*Bacillus coagulans* SNZ 1969" has been validated as per In- House protocols.

**For SANZYME BIOLOGICS (P) LIMITED**



**M.S. Muralidhar Goud**  
**Sr. Manager – QA**

**SANZYME BIOLOGICS (P) LTD**

**Regd. Office :** Plot No.13. Sagar Society, Road No.2,  
Banjara Hills, Hyderabad-500 034.,Telangana, India  
Phone : +91 40-4858 9999 | Fax : +91 40-4858 9913 |  
Website : [www.sanzymbiologics.com](http://www.sanzymbiologics.com) |  
email : probioticexports@sanzymbiologics.com  
CIN : U24110TG2016PTC112002

**Factory :** Plot Nos. 19 to 22, Sy. No's. 321/1,11,12,13 &  
276 & 277, Biotech Park, Phase - III, Genome Valley,  
Karkapatla (V), Markook (Mdl.), Siddipet (Dist.),  
Pin Code : 502 281. ,Telangana, India

**Factory :** Door No: 7-4-115, Sy. No: 258 & 259, Gaganpahad,  
Bangalore Road, Hyderabad – 500 052.,Telangana, India

**Table 5. Specifications of Bacillus coagulans SNZ 1969 preparation**

Parameter	Characteristics (Sanzyme Biologics, 2018)*	Method
Appearance	Dark grayish white powder	In- House
Identity	Aerobic, gram positive thermostable spores	In- House
Loss on drying (105° for 1 hour)	NMT5%	IP
Lactic acid producing capacity	Not Less Than 10 ml of 0.05 M NaOH is consumed	JPC
Viable spore	NLT 5 x 10 <sup>10</sup> spores/g	In- House
<b>Heavy metals</b>		
Arsenic	≤1mg/kg	ICP/OES
Lead	≤ 1mg/kg	ICP/OES
Mercury	≤ 1mg/kg	ICP/OES
Cadmium	≤ 1mg/kg	ICP/OES
<b>Microbiological assays</b>		
Total bacterial counts	NMT 0.1 million cfu/g	In- House
Yeast and Mold	NMT 10 cfu/g	USP 61
<i>Escherichia coli</i>	Negative/10 g	USP 62
<i>Salmonella</i>	Negative/10 g	USP 62
<i>Pseudomonas aeruginosa</i>	Negative/1 g	USP 62
<i>Staphylococcus aureus</i>	Negative/1 g	USP 62
<i>Bacillus cereus</i>	Negative/1 g	BAM Ch. 14
<i>Listeria monocytogens</i>	Negative/25 g	BAM Ch. 10
<i>Cronobacter sakazakii</i>	absent in 100 g	BAM Ch. 25