GRAS Conclusion for the Use of *Bacillus coagulans* SANK 70258 Spores Preparation (LACRIS-S) in Select Foods

SUBMITTED BY:

Mitsubishi-Kagaku Foods Corporation
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JAPAN

SUBMITTED TO:

U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
5001 Campus Drive
College Park, MD 20740

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February 3, 2017

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List of Acronyms

°C	degrees Celsius
µg	microgram
µm	micrometer
ADI	acceptable daily intake
bp	base pairs
BSL-1	biosafety level 1
bw	body weight
CAC	Codex Alimentarius Commission
cART	combination antiretroviral treatment
CFR	Code of Federal Regulations
CFU	colony forming unit
cGMP	current Good Manufacturing Practice
cm^2	centimeter squared
d	day
DNA	deoxyribonucleic acid
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
F	female
FDA	U.S. Food and Drug Administration
FOS	fructo-oligosaccharides
g	gram
GI	gastrointestinal
GRAS	Generally Recognized As Safe
GRN	GRAS Notice
h	hour
HIV	human immunodeficiency virus
kg	kilogram
M	male
mg	milligram
MKF	Mitsubishi-Kagaku Foods Corporation
mL	milliliter
n	number
NA	not applicable
NBRC	National Institute of Technology and Evaluation Biological Resource Center
NIH	National Institutes of Health
NLT	not less than
NMT	not more than

NOAEL	no observed adverse effect level
NOEL	no observed effect level
OECD	Organization for Economic Co-operation and Development
ppm	parts per million
QPS	qualified presumption of safety
RBC	red blood cell
RCT	randomized controlled trial
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	randomized trial
SD	Sprague Dawley
URI	upper respiratory infection
U.S.	United States
USDA	U.S. Department of Agriculture
WBC	white blood cell
wk	week
y	year

Part 1: Signed Statements and Certification

Mitsubishi-Kagaku Foods Corporation submits to the U.S. Food and Drug Administration (FDA) this generally recognized as safe (GRAS) notice in accordance with the 21 CFR part 170, subpart E.

Name and Address of Notifier

Mitsubishi-Kagaku Foods Corporation
1-1, Marunouchi 1-chome
Chiyoda-ku, Tokyo 100-8251, Japan

Name of GRAS Substance

The substance that is the subject of this GRAS notice is a preparation of *Bacillus coagulans* strain SANK 70258, a spore-forming lactic acid bacteria, diluted in lactose. The spores preparation is marketed under the trade name LACRIS-S.

Intended Use and Consumer Exposure

The proposed use of *B. coagulans* SANK 70258 in a spores preparation (up to 2×10^9 CFU/serving) includes use as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups. The proposed use of *B. coagulans* SANK 70258 in a spores preparation is identical to the use of four *B. coagulans* strains previously determined to be GRAS (GRNs 399, 526, 597, and 601) and therefore, would provide an alternate source of the microorganism in the spores preparation added to these foods. The estimated intake of *B. coagulans* strains from the proposed use was previously calculated at 36.4×10^9 CFU per day (GRN 399).

Basis for Conclusion of GRAS Status

Mitsubishi-Kagaku Foods Corporation's conclusion of GRAS status for the intended use of *B. coagulans* SANK 70258 in a spores preparation is based on scientific procedures in accord with 21 CFR §170.30(a) and (b).

Pre-Market Approval Exclusion Claim

Use of the preparation of *Bacillus coagulans* strain SANK 70258 is not subject to the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act because Mitsubishi-Kagaku

Foods Corporation has concluded that such use is generally recognized as safe (GRAS) through scientific procedures.

Availability of Information

The data and information that serve as the basis for this GRAS conclusion, as well as the information that has become available since the GRAS conclusion, will be sent to the FDA upon request, or are available for the FDA's review and copying during customary business hours at the office of Mary Murphy at Exponent, Inc., 1150 Connecticut Ave, NW, Suite 1100, Washington, DC 20036.

Exemptions from Disclosure

It is our view that none of the data and information in Parts 2 through 7 of the GRAS notice are exempt from disclosure under the Freedom of Information Act (FOIA). We redacted the following information from certain of the appendices for the reasons explained below:

- We redacted the identity of the "Client" in Appendix A-1 because it constitutes confidential commercial information and has no bearing on MKF's conclusion of GRAS status.
- We redacted the seals of the "Scientist Responsible" in Appendices A-1, A-2, A-3 and D, as well as the signature of the individual referenced in Appendix B, for reasons of personal privacy. That information has no bearing on MKF's conclusion of GRAS status.
- We redacted the name of an individual who is not employed by MKF in Appendix A-1 for reasons of personal privacy. That information has no bearing on MKF's conclusion of GRAS status.

Certification Statement

On behalf of Mitsubishi-Kagaku Foods Corporation, I hereby certify that, to the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(b) (6)

February 3, 2017

Name: Yukino Nagai
Title: Group Manager
Regulatory Affairs Group
Quality Assurance Department
Company: Mitsubishi-Kagaku Foods Corporation

Date

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

Identity

The substance that is the subject of this GRAS conclusion is a preparation of *Bacillus coagulans* strain SANK 70258, a spore-forming lactic acid bacteria, diluted in lactose. The preparation is marketed under the trade name LACRIS-S. Throughout this document, *Bacillus coagulans* strain SANK 70258 is to as *B. coagulans* SANK 70258 or as the *B. coagulans* strain in LACRIS-S.

Source of Organism and Use in a Spores Preparation

Bacillus species are spore-forming microorganisms naturally occurring in soil; they also are associated with production of enzymes, antibiotics and some chemicals, are used in some traditional ethnic fermented food such as natto, and may be present in food with germination of spores and subsequent growth potentially resulting in spoilage (Krawczyk, et al., 2016; Sanders et al., 2003).

The *B. coagulans* strain used in LACRIS-S, SANK 70258, was isolated from green malt in 1949 by a Japanese physician, Dr. Nakayama. The strain was identified in 1951 by Nakayama and colleagues as *Bacillus* coagulans Hammer (Nakayama et al., 1951). *B. coagulans* SANK 70258 spores preparation was launched by Sankyo Co., Ltd. (currently known as Daiichi Sankyo Co. Ltd.) in Japan under the trade name LACBON in 1964.

In 2007, Sankyo Co., Ltd. transferred the *B. coagulans* SANK 70258 strain, fermentation and formulation technology, and associated business to Mitsubishi-Kagaku Foods Corporation (MKF). Since then, it has been marketed in Japan, Taiwan and other countries under the brand name LACRIS.

In 1973, the Sankyo Corporation offered the formulation and fermentation technology of the *B. coagulans* strain to Sanzyme Ltd.; the strain was then designated strain SNZ1969 and was used to manufacture a spores preparation marketed under the trade name Sporlac® in India (Sanzyme, Ltd, 2015). *B. coagulans* SANK 70258 is therefore the mother strain of SNZ1969, a strain used in a spores preparation recently determined to be GRAS for use in select foods (Sanzyme, Ltd, 2015, GRN 597).

Description and Characterization of Organism

B. coagulans was first isolated and identified as the causative agent of coagulation of condensed milk in a condensary by B.W. Hammer at the Iowa Agricultural Experiment Station (Hammer, 1915). *B. coagulans* was independently identified in 1935 in the Fifth Edition of Bergey's Manual as *Lactobacillus sporogenes* since it shares characteristics of both genera. By the Seventh Edition of Bergey's Manual in 1957, the bacterium was reclassified in the *Bacillus*

genus (Reed et al., 1957). The bacterium may still occasionally be erroneously referred to as *L. sporogenes*.

Biochemical Characterization

The *Bacillus* genus was defined in 1920 as a genus of gram-positive, aerobic spore-formers, although 16S *ribosomal* RNA (rRNA) gene sequence analysis has resulted in an adjustment of this definition to include anaerobic and asporogenous members (Logan et al., 2011). In 2011, Logan and colleagues reported that the genus consisted of 162 species (Logan et al., 2011). Consistent with the genus description, *B. coagulans* SANK 70258 in LACRIS-S was demonstrated to be a gram-positive, aerobic to microaerophilic, endospore-forming rod, that was a L+ lactic acid producer, non-toxigenic and non-pathogenic, and approximately 0.9 µm x 3.0 µm x 5.0 µm in size (Kodama, 1992). The taxonomic characteristics of *B. coagulans* SANK 70258 are summarized in Table 1 along with characteristics of four other *B. coagulans* strains that have been the subject of GRAS notices (GRNs 399, 526, 597, 601).

Table 1. Taxonomic characteristics of *B. coagulans* SANK 70258 in LACRIS-S and other GRAS strains of *B. coagulans*

Characteristic	LACRIS-S[a] (SANK 70258)	GRN 399[b] Ganeden (GBI-30, 6086)	GRN 526[b] Unique Biotech (Unique IS2)	GRN 597[b] Sanzyme (SNZ1969)	GRN 601[b] Sabinsa (SBC37-01)
Origin	Green malt at a brewery		Human fecal soil	Green malt at a brewery	
Vegetative cells	Rods	Rods	Rods	Rods	
Gram staining	+	+	+	+	+
Cell size	0.9 x 3.0 x 5.0 µm	0.9 x 3.0 x 5.0 µm			
Motility	+			+	
Endospore formation	+	+	+	+	+
Shape of endospore	Oval	Oval	Oval		Rod-like
Cell distension in spore formation	+				
Position of spore	Terminal		Terminal		Terminal
Catalase	+		+	+	+
Growth under anaerobic conditions	+	Aerobic to microaerophilic	Aerobic to microaerophilic	Aerobic to microaerophilic	Aerobic to microaerophilic
Acid produced from sugar	D-glucose +		+		Glucose +
	D-xylose +		-	-	Xylose - variable
	L-arabinose +		+	-	Arabinose - variable
	D-mannitol -		+	-	Mannitol - variable
	Lactose +		+	-	
	Maltose +		+	+	
	Fructose +		+	+	
	Galactose +		+	+ (weak)	
Gas production from D-glucose	-		-		-
Indole test	-		-		-
Lactic acid production	+	+	+	+	+
Starch hydrolysis	+				+
Gelatin hydrolysis	+		-	-	
Nitrate reduction	+			-	Variable
Growth temperature	30 to 60°C		to 65°C	to 65°C	to 65°C

[a] Kodama, 1992

[b] Other strains have been determined to be GRAS for intended uses in foods and FDA was notified of the GRAS determinations (GRNs 399, 526, 597, 601)

Based on the microscopic observation and biochemical characterization of *B. coagulans* SANK 70258 in LACRIS-S summarized above, the bacterium identity as *B. coagulans* has been confirmed (Kodama, 1992). The microscopic observations and biochemical characterization of this strain of *B. coagulans* are consistent with characterization of *B. coagulans* in Ganeden Biotech's GRAS notice (GRN) of *B. coagulans* GBI-30, 6086 in 2011 (GRN 399), Unique Biotech's GRN of *B. coagulans* Unique IS2 in 2014 (GRN 526), Sanzyme Limited's (GRN 597) *B. coagulans* SNZ1969 in 2015 (GRN 597), and Sabinsa's *B. coagulans* preparation known as LactoSpore in 2015 (GRN 601), also shown in Table 1. MKF reports that *B. coagulans* SANK 70258 in LACRIS-S was deposited with the Institute of Applied Microbiology, University of TOKYO (IAM) in Japan as IAM 1146.

Genotypic Identification

In addition to microscopic observation and biochemical characterization for taxonomic characterization, molecular probing techniques using 16S ribosomal DNA (rDNA) primers and DNA-DNA hybridization testing have also been used to assess genetic identification.

16S rDNA Testing

16S rDNA analysis of a sample of *B. coagulans* SANK 70258 was performed by TechnoSuruga Laboratory Co., Ltd on two occasions (TechnoSuruga Laboratory Co., Ltd., 2014; Takaya, 2015; see Appendix A for reports).

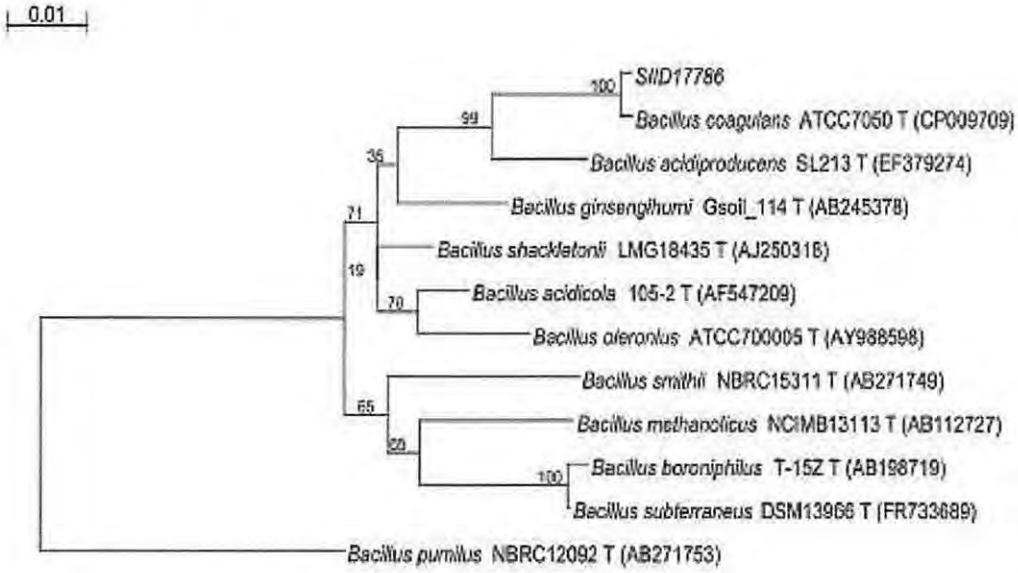
In the first analysis, reported on November 21, 2014, a partial sequence of the 16S rDNA gene (presumed to be 500 bp) was sequenced and compared to other known 16S rDNA gene sequences using two publicly available databases (TechnoSuruga Lab, 2014). The APOLLON DB-BA 10.0 BLAST database search identified the *B. coagulans* strain SANK 70258 as a *Bacillus* species with highest similarity to *Bacillus coagulans* (97.8%) strain NBRC12583. According to the NBRC catalogue, NBRC 12583 is listed as a *B. coagulans* Hammer 1915 type strain. In the GenBank/DDBJ/EMBL BLAST database search, a 99.2% similarity was reported with multiple *Bacillus coagulans* strains including strain SNZ1969 and undefined *Bacillus* species.

In the second analysis, reported on November 30, 2015, a 16S rDNA full sequence analysis was sequenced and compared to other known 16S rDNA gene sequences using the same databases as used in the previous year (TechnoSuruga Laboratory Co., Ltd., 2015). The APOLLON database was updated on August 15, 2015, and the GenBank database is updated every two months. *B. coagulans* SANK 70258 was identified as a *Bacillus* species with highest similarity to *B. coagulans* (99.3%) strain NBRC 12583 based on a APOLLON DB-BA 11.0 BLAST database search. In the GenBank/DDBJ/EMBL BLAST database search a 99.8% similarity was reported with multiple *B. coagulans* strains and undefined *Bacillus* species, and similarity to *B. coagulans* strain ATCC 7050 of 99.5 %.

A phylogenetic tree of *B. coagulans* SANK 70258 was constructed based on partial sequences of 16S rDNA of *B. coagulans* SANK 70258 and its related species in the 2014 report, and repeated in the 2015 report using the full 16SrDNA sequence. The two phylogenetic trees were similar, and the most recent phylogenetic tree, based on the full 16S rDNA sequence reported in 2015, is

included below (see Figure 1). Bootstrap values are indicated near each cluster and demonstrate a bootstrap value of 100 (rounded) between *B. coagulans* ATCC7050 T and *B. coagulans* SANK 70258 (SIID17786).

Figure 1. Phylogenetic tree of the *B. coagulans* strain SANK 70258 in LACRIS-S (SIID17786) and Related Species



DNA-DNA Hybridization Testing

A DNA-DNA hybridization analysis was performed on *B. coagulans* SANK 70258 and a reference strain of *B. coagulans, B. coagulans* NBRC12583 (TechnoSuruga Laboratory Co., Ltd, 2016). Results from the testing showed 55-64% homology between the SANK 70258 strain and the reference strain (TechnoSuruga Laboratory Co., Ltd., 2016; see Appendix A for report). This value is a relative one which shows distance of DNA sequences between a sample strain and a deposited type strain, and does not indicate a high degree of homology with the test strain. TechnoSuruga Laboratory Co., Ltd. noted in the report that DNA-DNA similarity values of more than 60% indicate species that could be defined as the same species, including subspecies. Published guidance indicates that DNA-DNA relatedness values should be around 70% or higher for designation of a species (Wayne et al., 1987).

A variety of techniques demonstrate a high degree of intra-species genomic diversity within *B. coagulans* strains, and the intra-species groupings are not consistent between different methods (De Clerck et al., 2004). As a consequence, the very heterogeneous nature of the species may result in significantly different results with differing methods to assess genetic identification (e.g. ARDRA, SDS-PAGE, FAME, 16srDNA and DNA-DNA hybridization).

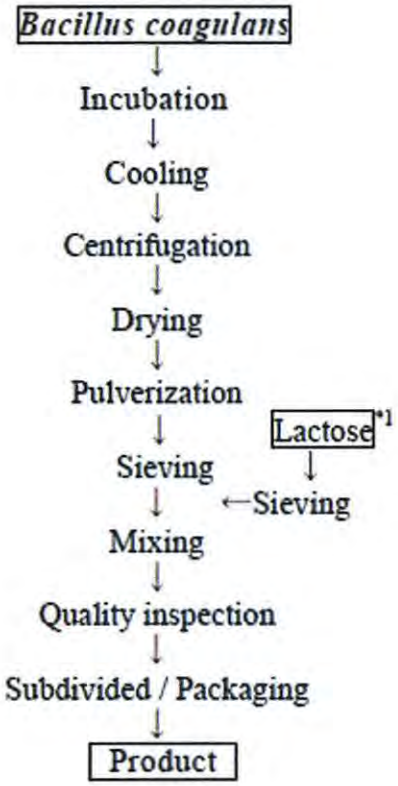
Summary of Characterization Data

Based on the microscopic observation, biochemical characterization and genotypic identification, *B. coagulans* SANK 70258 in LACRIS-S has been identified as *Bacillus coagulans*.

Method of Manufacture

The master cells are frozen at -80°C, and subculture is limited to up to two times to control the risk of variation. The prepared working cell is compared with the master cell morphologically and physiologically to check equivalency (i.e., bioequivalence test), and checked annually for genetic stability. The LACRIS-S preparation is made in a production process beginning with incubation of *B. coagulans* SANK 70258 followed by centrifugation, drying, mixing with a diluent, and packaging (see Figure 2). Time, temperature and pH are monitored at control points in the production process to ensure product quality. *B. coagulans* is manufactured in a dedicated line. During manufacture, microscopic checks are conducted to confirm the absence of morphological abnormity. The working cell of *B. coagulans* SANK 70258 is checked annually for genetic stability. MKF typically manufactures one lot of *B. coagulans*, from which up to 12 lots of LACRIS-S are generated. The manufacturer of *B. coagulans* is certified at Food Safety System Certification 22000 for manufacturing of the microorganism for use in food.

Figure 2. Manufacturing flow chart of LACRIS-S



Source: Mitsubishi-Kagaku Foods Corporation; March 27, 2015 *Milk origin

As previously noted, multiple lots of the LACRIS-S preparation are generated from one lot of *B. coagulans* SANK 70258. The additional raw materials used in the production of LACRIS-S include peptone, dextrose monohydrate, corn steep liquor, manganese sulfate pentahydrate, calcium chloride dehydrate, antifoaming agent, yeast extract, lactic acid, lactose. All substances used in the manufacture of LACRIS-S are commonly used in the manufacture of fermented foods and are used in accord with current good manufacturing practice (cGMP). Analytical data from three lots of *B. coagulans* are summarized in Table 2; complete analytical reports are provided in Appendix B.

Table 2. Analytical results for Lot Analyses of *Bacillus coagulans* SANK 70258

Parameter	Unit	Lot Number, Date of Manufacture[a]			Method of Analysis
		141004AA	150221AA	150307AA	
Description	NA	Light brown powder, slightly unique odor	Light brown powder, slightly unique odor	Light brown powder, slightly unique odor	visual
Identification	NA	Conforms	Conforms	Conforms	JSPI
Loss on drying	%	7.8	5.7	6.3	JSSFA
B. coagulans spore count	CFU/g	5.09×10^{11}	6.05×10^{11}	4.88×10^{11}	Int method
Heavy Metals					
Arsenic	ppm	nd (<0.1)	0.1	0.1	JSSFA As Method 3
Cadmium	ppm	0.02	0.01	0.02	JSSFA AAS
Lead	ppm	nd (<0.05)	nd (<0.05)	nd (<0.05)	JSSFA AAS
Mercury	ppm	nd (< 0.01)	nd (<0.01)	nd (<0.01)	JSSFA AAS
Potential Contaminants					
Total bacteria (SCDA)	CFU/g	< 10	<10	<10	JFSA
Coliforms	/g	Negative	Negative	Negative	JFSA
Fungus	CFU/g	< 10	<10	<10	JFSA
E. coli	NA	Negative/2.22 g	Negative/2.22g	Negative/2.22g	JFSA
Salmonella	/25 g	Negative	Negative	Negative	JFSA
Listeria monocytogenes	/25g	Negative	negative	Negative	JFSA
Enterobacteriacea	CFU/g	< 10	<10	<10	JFSA

[a] Analytical reports are provided in Appendix B

CFU – colony forming unit, JFSA - Japanese Food Sanitation Act; JSPI – Japanese Standards for Pharmaceutical Ingredients; JSSFA – Japan's specifications and standards for foood additive; NA – not applicable; nd – not detected; ppm – parts per million; SCDA – soybean casein digest agar

Specifications

Analytical data from six lots of LACRIS-S prepared from three lots of *B. coagulans* SANK 70258 are summarized in Table 3; complete analytical reports for LACRIS-S are provided in Appendix B along with details of the analytical methods. These specifications include conformation with identity, minimum *B. coagulans* spore counts, and limits for moisture, lactic acid production, heavy metals and potential microbiological contaminants including total bacteria, fungus, and coliforms (see Table 3).

Table 3. Product specifications and Lot Analyses of LACRIS-S

Parameter	Unit	Specification	Lot Number, Date of Manufacture[a]						Method of Analysis
			A11015 11/25/2014	B10238 3/24/2015	B10240 3/25/2015	B11299 12/17/2015	B11201 12/17/2015	C10103 1/26/2016	
Description		White to yellow powder, unique odor	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Visual, Olfactory
Identification	NA	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	JSPI
Loss on drying	%	NMT 8.0	1.1	2.1	2.5	1.7	1.7	3.4	JSSFA
Lactic acid producing ability	mL	NLT 10	20	19	19	19	19	19	Int. method
B. coagulans spore count	CFU/g	NLT 5.0×10^9	7.3×10^9	7.1×10^9	6.4×10^9	6.1×10^9	6.2×10^9	10.0×10^9	Int. method
Heavy Metals									
Arsenic	ppm	NMT 0.5	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	JSSFA As Method 3
Cadmium	ppm	NMT 0.5	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	JSSFA AAS
Lead	ppm	NMT 0.5	nd (<0.05)	nd (<0.05)	nd (<0.05)	nd (<0.05)	nd (<0.05)	nd (<0.05)	JSSFA AAS
Mercury	ppm	NMT 0.5	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	nd (<0.01)	JSSFA AAS
Potential Contaminants									
Total bacteria	CFU/g	NMT 1,000	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	JFSA
Fungus	CFU/g	NMT 500	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	JFSA
Coliforms	/g	Negative	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	JFSA

[a] COAs are provided in Appendix B.

AAS – Atomic absorption spectrometry; Int. method – internal method; JFSA – Japanese Food Sanitation Act; JSPI – Japanese Standards for Pharmaceutical Ingredients; JSSFA – Japan's specifications and standards for foood additive; CFU – colony forming unit; nd – not detected; NLT – not less than; NMT – not more than; ppm – part per million

Shelf-Life Stability

A shelf-life stability testing was performed to assess the stability of *B. coagulans* SANK 70258 in LACRIS-S. The test was performed over a seven-month period using LACRIS-S product lot number XA11015. Stability was assessed at room temperature and above (approximately 20 - 35°C) and a relative humidity of 25-60%. After seven months, approximately 97% of the *B. coagulans* remained viable in the LACRIS-S (see Table 4).

Table 4. Shelf-Life Stability Test of *B. coagulans* in LACRIS-S

Time since manufacture (months)	**Initial *B. coagulans* spore count /g of LACRIS-S**	**Percentage residual *B. coagulans***
0	7.3×10^9	100
7	7.1×10^9	97

Shelf-life stability test was conducted on LACRIS-S in a dietary supplement for 7 months. The initial spore count of *B. coagulans* was 3.3×10^8 CFU/g and after 7 months the spore count was 1.8×10^8 CFU/g, equivalent to 55% residual. A comparable shelf-life stability test under similar conditions was conducted on LACRIS-S in hard candy over a period of six months. The initial spore count of *B. coagulans* was 1.3×10^7 CFU/g; by three months the spore count declined to 7.1×10^6 CFU/g (55% residual) and by six months the spore count declined to 6.9×10^6 CFU/g (53% residual). Results from these two tests indicate comparable stability of *B. coagulans* in dietary supplement and hard candy matrices.

Technical Effect

The preparation of *Bacillus coagulans* strain SANK 70258 is proposed for addition to food for a nutritive effect, i.e., providing a dietary source of the microorganism, rather than a technical effect.

Part 3. Dietary Exposure

Food Uses in the United States

B. coagulans is recognized as GRAS (21 CFR §184.1372) in the production of insoluble glucose isomerase enzyme preparations. Specifically, the U.S. FDA states that insoluble glucose isomerase enzyme preparations derived from nonpathogenic and nontoxicogenic microorganisms, including *B. coagulans*, may be used as ingredients in food with no limitation other than cGMP. *B. coagulans* is classified by ATCC as a Biosafety Level One (BSL-1) organism based on U.S. Public Health Service Guidelines, indicating the organism is not known to cause disease in healthy human adults.

Spores preparations of *B. coagulans* have been the subject of four (4) GRAS notices (GRN 399, 526, 597, 601). The U.S. Food and Drug Administration (FDA) had no questions concerning Ganeden Biotech's conclusion that a preparation of *B. coagulans* strain BBI-30, 6086 spores was GRAS under the intended conditions of use (GRN 399; FDA 2012) at a maximum level of approximately 2 x 10^9 CFU per serving (CFU/serving) as an ingredient in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups.

More recently, the FDA had no questions concerning Unique Biotech Limited's conclusion that a spores preparations of *Bacillus coagulans* Unique IS2 as well as Sanzyme Limited's conclusion that a spores preparation of *Bacillus coagulans* SNZ1969 are GRAS under the intended conditions of use (GRN 526; FDA 2015, and GRN 597, FDA 2016). The intended uses of the spores preparations of *B. coagulans* Unique IS2 and SNZ1969 are identical to the proposed uses of the preparations of *B. coagulans* GBI-30, 6086 spores, namely a maximum level of approximately 2 x 10^9 CFU/serving in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups.

Sabinsa Corporation also concluded that their *B. coagulans* SBC37-01 spores preparation, also known as LactoSpore®, is GRAS for the same intended uses (Sabinsa Corporation, (GRN 601) 2015) and this was accepted by FDA although at this time, FDA has not yet posted a letter to the GRN Inventory stating they have no objection to this GRAS determination (Sabinsa Corporation, 2016).

Supplement Uses in the United States

B. coagulans is marketed in the United States as a dietary supplement for human consumption. General internet searches and a search of the NIH Dietary Supplement Label Database (http://dsld.nlm.nih.gov/dsld/) show various *B. coagulans*-containing dietary supplements on the market, with recommended doses for *B. coagulans* typically on the order of 10^8 to 10^9 CFU per serving, to be consumed one or more times per day. The use of *B. coagulans* as a dietary supplement is also noted in a previous GRAS notice for *B. coagulans* Unique IS2 (GRN 526).

International Uses

Use of *Bacillus* species has been documented for over 50 years with the introduction of products such as Enterogermina™ in Italy in 1958 (Cutting et al., 2010). *B. coagulans* SANK 70258 spores preparation was launched by Sankyo Co., Ltd. (currently known as Daiichi Sankyo Co. Ltd.) in Japan under the trade name LACBON in 1964. During 1964 and 1965, *B. coagulans* was investigated in several small clinical studies in Japan. In 1972, at the request of Sankyo Co., Ltd., the Japanese Ministry of Health and Welfare approved the use of this particular *B. coagulans* SANK 70258. *B. coagulans* SANK 70258 spores preparation was also launched by Sankyo Co., Ltd. in Japan as a probiotic in food categories under the trade name LACRIS in 1966. As summarized by Sanzyme Ltd. (2015), *B. coagulans* has been approved and marketed in India under the brand name Sporlac for the past four decades. *B. coagulans* also is reported to be among the microorganisms used in the fermentation of the African bean oil to make ugba, a traditional food consumed in Nigeria (Isu and Njoku, 1997).

The European Food Safety Authority (EFSA) granted Qualified Presumption of Safety (QPS) status of *B. coagulans* in the absence of toxigenic activity and the absence of acquired antimicrobial resistance genes to clinically relevant antibiotics and that status remains, which indicates that use of *B. coagulans* in foods is considered safe (EFSA, 2008; 2015). In relation to Standard 1.5.1, the Food Standards Australia New Zealand views *Bacillus coagulans* as a non-traditional though not novel food, citing some evidence of use in natto and no identified safety concerns (FSANZ, 2015). *B. coagulans* is listed in the Codex Alimentarius Commission's list of food for the production of insoluble enzyme preparations (1987). Health Canada (2015) also recognizes no limitations other than good manufacturing practice for the use of *B. coagulans* in the production of insoluble glucose isomerase preparations (isomerization of glucose to fructose).

Proposed Use and Level

B. coagulans SANK 70258 in the LACRIS-S spores preparation is proposed for use (up to 2 x 10^9 CFU/serving) as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices;

snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups. The *B. coagulans* SANK 70258 spores preparation will not be used in any foods for which food standards would preclude its use.

The proposed use of LACRIS-S, resulting in a concentration of *B. coagulans* SANK 70258 of up to 2 x 10^9 CFU/serving as an ingredient in select food categories, is identical to the proposed use of spores preparations containing *B. coagulans* GBI-30, 6086 (GRN 399), *B. coagulans* Unique IS2 (GRN 526), *B. coagulans* SNZ1969 (GRN 597), and *B. coagulans* SBC37-01 which is known as LactoSpore® (GRN 601).

Estimated Daily Intakes

Given that the proposed use of *B. coagulans* SANK 70258, including both the maximum use level of up to 2 x 10^9 CFU/serving and the specific food categories in which it will be used as an ingredient are identical to use of four other GRAS sources of *B. coagulans* (GRNs 399, 526, 597, 601), it is reasonable to assume that use of *B. coagulans* SANK 70258 provides an alternate source of this organism. The total intake of *B. coagulans* resulting from use of *B. coagulans* SANK 70258 therefore is likely to be unchanged from current intake of *B. coagulans.*

In the GRAS Notice for *B. coagulans* GBI-30, 6086 (GRN 399), Ganeden calculated the maximum estimated daily intake (EDI) of the organism from the intended use of up to 2 x 10^9 CFU/serving in the United States at 36.4 x 10^9 CFU per day. This estimate was based on data from the USDA Center for Nutrition Policy and Promotion showing that the maximum number of food servings consumed per day is 18.2 (among the population of males ages 51 years and older) and the assumption that the maximum intended use of the organism would be added to each serving of food consumed. The FDA had no questions regarding the proposed use levels and the resulting EDI as calculated by Ganeden (GRN 399) or in use of a substitutional approach as proposed in subsequent GRAS Notices for identical uses of a *B. coagulans* spores preparation (GRN 526, GRN 597, GRN 601).

Since the proposed use of *B. coagulans* SANK 70258 is substitutional for use of preparations of *B. coagulans* spores from strains GBI-30, 6086, Unique IS2, SNZ1969 or LactoSpore®, the maximum EDI of all strains of *B. coagulans* combined is 36.4 x 10^9 CFU per day.

Part 4. Self-Limiting Levels of Use

B. coagulans SANK 70258 in the LACRIS-S spores preparation is proposed for use up to 2×10^9 CFU/serving. We are not aware of technological or palatable issues associated with the proposed use levels. Self-limiting levels of use are not applicable to this notice.

Part 5. Experience Based on Common Use in Food before 1958

The conclusion of GRAS status of the use of a preparation of *Bacillus coagulans* strain SANK 70258 in foods was based upon scientific procedures. Experience based on common use in food before 1958 is not applicable to this notice.

Part 6. Narrative

Introduction

LACRIS-S is the trade name for a proprietary preparation of *Bacillus coagulans* strain SANK 70258, a spore-forming lactic acid bacteria. The LACRIS-S preparation consists of spores of *B. coagulans* diluted in lactose.

The proposed use of *B. coagulans* SANK 70258 in a spores preparation (up to 2 x 10^9 CFU/serving) includes use as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups. The proposed use of *B. coagulans* SANK 70258 in a spores preparation is identical to the use of the *B. coagulans* strains previously determined to be GRAS and therefore, would provide an alternate source of the microorganism in the spores preparation added to these foods. The estimated intake of *B. coagulans* strains from the proposed use was previously calculated at 36.4 x 10^9 CFU per day. The FDA had no questions regarding the proposed uses and use levels and the resulting EDI as calculated originally by Ganeden (GRN 399) or in use of a substitutional approach as proposed in three subsequent GRAS Notices for identical uses of a *B. coagulans* spores preparation (GRNs 526, 597, 601).

The safety of the proposed use of *B. coagulans* SANK 70258 in the spores preparation marketed under the name LACRIS-S was evaluated based on a review of the totality of 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. Available information included evidence and data provided in four safety reviews previously developed to establish the GRAS status of the use of other *B. coagulans* strains (GRNs 399, 526, 597, and 601), 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 provided by MKF on the phenotypic and genotypic analysis of the organism. Information on the established history of safe use of a microorganism is also an important consideration in a safety evaluation of a strain, and when available can negate the need to follow a more thorough decision tree approach for determining safety of microbial cultures (Pariza et al., 2015). The totality of evidence, in combination with information on the established history of use of the species, was relied upon to determine the safety of the strain in a spores preparation.

Identification of the Microorganism

Bacillus species are potential spore-forming microorganisms that are naturally occurring in soil and *Bacillus coagulans* species are used in the fermentation of ethnic foods such as ugba in

Nigeria. Data from conventional microscopic observation and biochemical characterization in combination with genotypic identification, including a 16S rDNA full sequence analysis and comparison to known gene sequences, provide evidence that the strain used in the production of the LACRIS-S spores preparation and referred to as strain SANK 70258 is of the *Bacillus coagulans* species. The *B. coagulans* strain in LACRIS-S was identified as a *Bacillus* species with similarity to known *Bacillus coagulans* strains of 99.5% (with ATCC 7050) and 99.8% (with multiple other strains) in a GenBank/DDBJ/EMBL BLAST database search of the full 16S rDNA sequence. The *B. coagulans* SANK 70258 strain is routinely assessed with morphological and physiological tests to ensure consistency, it is examined annually for genetic stability, and microscopic checks throughout the production process are conducted to confirm the absence of morphological abnormity.

All substances used in the manufacture of the LACRIS-S preparation are commonly used in the manufacture of fermented foods and are used in accord with current good manufacturing practice (cGMP). The LACRIS-S preparation is made in a production process with controls to ensure suitability for use in food and the product meets specifications appropriate for a food ingredient. The LACRIS-S preparation contains a minimum *B. coagulans* spores count of 5.0×10^9 CFU per g.

Use of *B. coagulans* dates back more than 50 years with the introduction of the *B. coagulans* SANK 70258 strain in a spores preparation for probiotic foods. More recently, use of spores preparations developed from several strains of *B. coagulans* has been determined to be GRAS for use in a wide range of foods as summarized in GRAS Notices (GRN) 399, 526, 597, and 601. In addition to the recognition of GRAS uses in foods, *B. coagulans* has been granted qualified presumption of safety (QPS) status which suggests that use of *B. coagulans* in foods is considered safe (EFSA, 2008; 2015), FSANZ identified no safety concerns (FSANZ, 2015), and *B. coagulans* may be used for the production of insoluble enzyme preparations (CAC 1987; Health Canada, 2015; 21 CFR §184.1372).

Antibiotic Resistance and Potential Production of Virulence Factors

Antibiotic Susceptibility

Antibiotic susceptibility of *B. coagulans* SANK 70285 spores preparation was assessed using both the disk diffusion method and the microdilution method (Sakuma, 2016; see Appendix C). The disk diffusion method examines the mean diameter of zone of growth inhibition around an antibiotic disc. Antibiotics tested with the disk diffusion method were: streptomycin, gentamicin, bacitracin, novobiocin, polymixin, cefaclor, ciprofloxin, rifampicin, chloramphenicol, tetracycline, erythromycin, kanamycin, colistin, nalidixic acid, clindamycin, cefoxitin, doxycyline, and penicillin. Using the disk diffusion method, the *B. coagulans* SANK 70285 spores preparation was determined to be sensitive to all 18 antibiotics tested.

The microdilution method determines the lowest concentration (i.e., minimum inhibitory concentration or MIC) of an antibiotic that inhibits bacterial growth. Antibiotics tested with the microdilution method were: oxacillin, ampicillin, cefazolin, cefmetazole, flomoxef, imipenem, gentamicin, arbekacin, minocycline, cefoxitin, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, fosfomycin, sulfamethoxazole-trimethoprim, and levofloxacin. With the

exception of flomoxef and linezolid, bacterial growth was inhibited with the lowest concentration of each antibiotic tested. The MICs for flomoxef and linezolid were 1.0 μg/mL and 0.5 μg/mL, respectively.

The *B. coagulans* SANK 70285 spores preparation was concluded to be susceptible to all antibiotics tested using the disk diffusion method and the microdilution method. Previously reported analyses of *B. coagulans* also demonstrate that strain SNZ1969, a strain of *B. coagulans* closely related to *B. coagulans* SANK 70258, is susceptible to numerous antibiotics; resistance was noted for a limited number of tested antibiotics including cefuroxine and metronidazole using the Hexa Discs method, and cefaclor, cefoxitin, colistin, novobiocin, and metronidazole using the agar disk diffusion method (Sanzyme Ltd., 2015). Results from genomic analysis of the Sanzyme Ltd. strain showed no evidence for the presence of plasmids, suggesting the inability to gain and transfer plasmid-borne antibiotic-resistance genes in the strain (Khatri et al., 2016). Since SNZ1969 is the daughter strain of SANK70258 and thus both strains originated from the same source, the strains would likely share similar antibiotic resistance profiles. 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). Both the SANK70258 strain and the SNZ1969 strain are maintained in pure culture, and at least for SANK70258, kept frozen and without the presence of antibiotics. As such, it is unlikely that additional antibiotic resistance has been acquired by the SANK70258 strain since 1973 when the two strains were split.

Additional studies provide evidence that other strains of *B. coagulans* are susceptible to a range of common antibiotics. Using a disk diffusion method, Sudha and colleagues (2010) demonstrated that *B. coagulans* Unique IS2 was sensitive to Cefaclor, Cephoxitin, Chloramphenicol, Ciprofloxacin, Gentamycin, Kanamycin, Nalidixic acid, Polymixin B, Rifampcin, Trimethoprim, and Novobiocin while it displayed intermediate sensitivity to Clindamycin, Doxycycline, Erythromycin, Penicillin, and Tetracycline, and resistance to Bacitracin, Colistin, Methicilin, Metronidazole, and Streptomycin. As summarized by Sanzyme Ltd. (2015), Moldenhauer and colleagues (1996) published findings that *B. coagulans* ATCC 51232 was susceptible to 28 of 30 antibiotics testing in a study in which zones of inhibition of growth were evaluated.

Virulence Activity

An important area of concern with probiotic use is infectivity and pathogenicity. Notwithstanding the known and significant potential of *Bacillus anthracis* as a human pathogen and *Bacillus cereus* as a food poisoning agent, the majority of *Bacillus* species strains rarely cause infection in humans (Tortora et al., 2007), and those infections that do occur do so in individuals with weakened immune systems (Logan et al., 2011, Sanders et al., 2003). A study by Banerjee and colleagues (1998) in which 24 episodes of *Bacillus* bacteremia was reported in 18 cancer patients, with only one episode identified as related to *B. coagulans*, suggests that *B. coagulans* is likely only an opportunistic bacterium in highly immunocompromised populations. Other studies of *B. coagulans* in immunocompromised populations including adults with HIV, full-term infants, and premature infants have reported no significant adverse effects (Chandra et al., 2002; Sari et al., 2011; Yang et al., 2014). Sari and colleagues specifically noted that the incidence of sepsis did not significantly differ between treatment groups. Clinical infections

reported for *B. coagulans* are typical opportunistic infections and were isolated from corneal infection and bacteremia (Logan et al., 2011). No information was identified indicating that oral ingestion of *B. coagulans* causes infection.

A cereus enterotoxin detection test (Duopath® Cereus Enterotoxin test kit, Merck) was completed on a sample of *B. coagulans* SANK 70258 (TechnoSuruga Laboratory Co., Ltd., 2016b; see Appendix D). The organism was concluded to be negative for non-hemolytic enterotoxin and hemolysin BL enterotoxin.

Summary

In summary, *B. coagulans* is susceptible to a variety of common antibiotics as demonstrated in published (Moldenhauer et al., 1996; Sudha et al., 2010) and unpublished studies. Genetic testing on *B. coagulans* SNZ1969, a strain of *B. coagulans* closely related to *B. coagulans* SANK 70258, indicates that the strain showed no evidence for the presence of plasmids and consequently is not likely to gain and transfer plasmid-borne antibiotic-resistance genes (Khatri et al., 2016). No information was identified indicating that oral ingestion of *B. coagulans* causes infection, and testing of the *B. coagulans* SANK 70258 strains demonstrates that the strain is negative for non-hemolytic enterotoxin and hemolysin BL enterotoxin.

Pre-Clinical and Clinical Evidence of Safety

Approximately 4 hours after ingestion, the microorganism passes into the duodenum and over approximately a one-week period following ingestion the spores are excreted. The available data therefore indicate a transient nature of *B. coagulans* in the gastrointestinal tract without any bioaccumulation. *B. coagulans* is classified as Biosafety Level 1(BSL-1) organism, thus indicating the organism is not known to cause disease in healthy human adults. *B. coagulans* SANK 70258 and several similar strains have been tested in a range of pre-clinical and clinical studies. The *B. coagulans* strains tested in pre-clinical and clinical studies are very similar to *B. coagulans* SANK 70258 and these data can be used to support the safety of *B. coagulans* SANK 70258 in a spores preparation. These studies are briefly summarized below in and in Tables 5 and 6.

Published Pre-Clinical Data on *B. coagulans*

Genotoxicity Data

The mutagenicity or clastogenicity of *B. coagulans* – GanedenBC30™ (*B. coagulans* GBI-30, 6086) was tested in an *in vitro* bacterial reverse mutation assay (OECD 471); *in vitro* chromosomal aberration assay and micronucleus assay in mice (Endres et al., 2009). The results of these studies indicate that GanedenBC30TM *B. coagulans* did not demonstrate mutagenic, clastogenic, or genotoxic effects under the conditions of the experiments.

Acute Oral Toxicity

The acute oral toxicity of *B. coagulans* Unique IS2 (MTCC 5260 (5×10^9 CFU/g) was investigated in Sprague Dawley (SD) rats (n=6/sex/group) at three doses (0 (control, vehicle), 3,250 and 6,500 mg/kg body weight (mg/kg bw)). The results of this study after 14 days of observation show that the LD_{50} of *B. coagulans* Unique IS2 is greater than 6,500 mg/kg bw (32.5×10^9 CFU /kg bw) (Sudha et al., 2011a). In another oral acute toxicity study, a single oral dose of *B. coagulans* GBI-30, 6086 cell mass was administered to Wistar rats (n = 5/sex) at a dose of 5,000 mg/kg bw (5.2×10^{11} CFU /kg bw). The results of this study suggested that the LD_{50} of the cell mass containing *B. coagulans* was greater than 5,000 mg/kg bw (5.2×10^{11} CFU /kg bw) (Endres et al., 2009). Overall, the oral LD_{50} for different strains of *B. coagulans* range from 5,000 – 6,500 mg/kg bw.

Short-term Oral Toxicity

The short-term, repeat-dose oral toxicity of *B. coagulans* Unique IS2 was investigated in SD rats (6/sex/group) (Sudha et al., 2011a). Rats were administered orally (gavage) with 0 (Group I), 130 (Group II), 650 (Group III) or 1300 mg (group IV) *B. coagulans* Unique IS2 preparation/kg body weight/day (kg bw/d) for 14 consecutive days. The *B. coagulans* Unique IS2 preparation contained 5×10^9 CFU/g, resulting in daily dose of *B. coagulans* Unique IS2 for each group of 0.0, 0.65×10^9, 3.25×10^9 and 6.50×10^9 CFU/kg for Groups I, II, III and IV, respectively. All animals were observed daily for clinical signs and mortality. Body weights and detailed clinical examinations were recorded weekly. Urine analysis was performed during the last week of the study and hematological analysis and biochemical analysis were performed on the day of necropsy. Half of the animals from each group were sacrificed on day 15, while remaining animals (recovery group) were euthanized on day 28 and observed for gross lesions. Histopathological examination was conducted on standard organs for histopathology. There were no treatment-related changes reported in clinical signs, body weights, feed intake, urine parameters, hematological parameters, clinical chemistry, gross pathology and histopathology. The authors concluded that *B. coagulans* Unique IS2 was clinically well tolerated at doses up to 1300 mg or 6.5×10^9 CFU/kg bw/d, when administered orally to SD rats for 14 days consecutively. The No Observed Adverse Effect Level (NOAEL) for *B. coagulans* Unique IS2 was established as the highest dose tested, 1,300 mg (6.5×10^9 CFU)/kg bw/d.

Gu et al., (2015) investigated the short-term oral toxicity of a new strain of *B. coagulans* CGMCC 9551, isolated from piglet feces. Groups of KM mice (a group consists of 5 males and 6 females) were administered a saline suspension of *B. coagulans* CGMCC 9951 at 1×10^6, 1×10^8, and 1×10^{10} spores/kg bw/d via gavage daily for 4 weeks. The animals were observed daily for clinical signs of toxicity and mortality. Daily feed intake and body weight of mice were measured. After 4 weeks of administration, the animals were sacrificed and their heart, liver, spleen, lungs, kidneys, thymus, testes, ovaries were removed and weighed. Hematological and clinical chemistry parameters were not measured and histopathological examination of organs was not conducted in this study. No morbidity and mortality were reported in the treatment groups. The organ weights of heart, liver, spleen, lungs, kidneys, testes and ovaries of the treatment groups were not significantly different from that of the control groups, except for the spleen and thymus. There was a statistically significant increase in spleen weight in males and females of the mid dose (1×10^8) and high dose (1×10^{10}) groups. There was also a statistically

significant increase in thymus weight in females of the mid-dose and high-dose groups and in all treated males. According to the authors, these data suggest that *B. coagulans* CGMCC 9951 may be involved in the modulation of the immune system of KM mice. Feed intake and body weight gains of treatment groups increased considerably compared with the control group (data not shown in this paper). The authors concluded that the results from this study indicate that CGMCC 9951 was safe at a dose up to 1 X 10^{10} spores/kg bw/d.

Subchronic Oral Toxicity

The subchronic oral toxicity potentials of GanedenBC30™ (*B. coagulans* GBI-30, 6086) was investigated in Wistar rats according to OECD 408 guidelines and the FDA Redbook (Endres et al., 2009). *B. coagulans* (GanedenBC30™) cell mass (1.36 x 10^{11} CFU/g) was orally administered (gavage) to Wistar rats (10/sex/group) at doses of 0, 100, 300 and 1,000 mg/kg bw/d for 90 consecutive days. The highest dose treated animals received a dose of 1.36 x 10^{11} CFU *B. coagulans*/kg bw/d.

Clinical observations were made once daily following treatment at approximately the same time each day. Detailed clinical observations were made on all animals prior to the first exposure, and once a week thereafter. Body weight was measured on day one, once weekly thereafter, and on the day of autopsy. Food was weighed weekly, and the average food consumption per animal was calculated. Water consumption was measured over a 24-h period weekly, and average water consumption per animal was calculated. During week 13 of administration, sensory reactivity to different types of stimuli (auditory, visual and proprioceptive) was measured, as well as general physical condition and behavior of animals. Functional observational battery was performed using the modified Irwin test (Irwin, 1968). Hematological parameters and clinical chemistry analyses were evaluated at the end of the study from animals that were fasted for 16 h prior to blood collection. Animals were sacrificed and gross pathological examination was performed on all organs, including organ weight and appearance. A histopathological analysis was performed on the control group and the highest dose group, and on the middle dose groups when appropriate.

No deaths or treatment-related signs were reported throughout the study in any of the groups. Appearance and behavior of the animals were similar for all groups. There were some statistically significant differences in some parameters, such as a lower water consumption in females at all doses levels and in some hematological and clinical chemistry parameters. However, there were no toxicologically significant differences between the treatment and control groups in feed consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations. At study termination, no treatment-related macroscopic or microscopic (histopathological) changes in the organs were reported. The absolute brain, liver and testes weight were lower at 1,000 mg/kg bw/d; 100 and 1,000 mg/kg bw/d and 100 mg/kg bw/d, respectively. However, the relative organ weights for these organs did not differ between treatment and control groups. The relative kidney weight was lower in the males at 300 mg/kg bw/d, and higher in the males in the 1,000 mg/kg bw/d dose. The changes were not considered treatment-related or of biological significance because they were not accompanied by any histological findings. The relative weight of the adrenal glands was lower than the control group for the females in the 300 mg/kg bw/d, but was due to individual variation and not treatment-related because of a lack of dose-response. The authors concluded that GanedenBC30TM did not cause treatment-related macroscopic or microscopic signs or changes

in the organ weights of the male and female rats at 100, 300 and 1,000 mg/kg bw/d after the 13-week treatment period. The test item was well tolerated. The NOAEL for both males and females was determined to be >1,000 mg (1.36×10^{11} CFU)/kg bw/d, the highest dose tested (Endres et al., 2009).

Chronic Oral Toxicity

The safety of long-term consumption of *B. coagulans* – GanedenBC30™ (*B. coagulans* GBI-30, 6086) was investigated in a one-year chronic oral toxicity study combined with a one-generation reproduction study (described below) (Endres et al., 2011). This study was conducted in accordance with OECD Guideline for the Testing of Chemicals, No. 452 (12 May 1981) and US FDA Redbook 2000 standards IV.C.5.a., ''Chronic Toxicity Studies with Rodents'' (July 2007).

Male and female HsdBrlHan rats of Wistar origin (n=20/sex/group) were administered GanedenBC30™ in rat feed at 0, 10,000, 20,000 and 33,300 mg/kg (equivalent to 0, 600, 1,200 and 2,000 mg/kg bw/d) for 52 – 53 weeks 7 days a week. Observations for signs of morbidity and mortality were made twice daily, and general clinical observations were conducted once daily following treatment. Detailed clinical observations were made on all animals before the first exposure, and once a week thereafter. Body weight was measured on day one, once weekly for 13 weeks, and once every 4 weeks hereafter. Food was weighed weekly, and the average food consumption per animal was calculated. Ophthalmological examination was performed prior to study initiation and at the end of the study. Clinical pathology examinations, including hematology and chemistry analyses were conducted in all animals at the end of the first 3 weeks, and at 3, 6, and 12 months of study. Urinalysis was performed the week prior to initiation of treatment, and at the end of study months 3, 6, and 12. Liver, brain, heart, thymus, spleen, kidneys, testes, epididymis, uterus, thyroid/parathyroid, adrenal glands and ovaries weights were measured. Full histopathological examination was performed in the control (0 mg/kg bw/d) and high dose (2,000 mg/kg bw/d) groups, and in animals found dead or moribund. Any observations of abnormalities in organs from the mid- and low-dose groups resulted in histopathological examination of the relevant tissues.

The actual doses of GanedenBC30™ in the various dose groups was analyzed from dietary intake to be as follows: at 600 mg/kg bw/d target dose group, males received a calculated mean daily intake of 585 mg/kg bw/d and females received 761 mg/kg bw/d; at 1,200 mg/kg bw/d target dose group, males received 1,147 mg/kg bw/d and females received 1,467 mg/kg bw/d); and at 2,000 mg/kg bw/d target dose group, males received 1,951 mg/kg bw/d and females received 2,525 mg/kg bw/d.

No mortality was reported in any of male treatment groups. One female treated with 1,200 mg/kg bw/d was found dead on day 137, but this was attributed to an individual disorder. Clinical observations did not reveal any toxic signs related to the test article. The signs and symptoms occurring mainly in the females were considered individual alterations, as these signs are often seen in experimental rats and were not dose-related. Ophthalmoscopic changes reported were not treatment-related.

There were no treatment-related changes in body weight, body weight gain, or feed consumption during the study. No toxicologically relevant changes in hematological, clinical chemistry or urine parameters were reported at 3 weeks and at 3, 6 or 12 months. Statistically significant changes noted were either not dose-related or were well within the historical background range

or were not accompanied by other hematological or histopathological changes or a single occurrence or of low magnitude. At termination, macroscopic and microscopic examinations did not reveal treatment-related lesions. The NOAEL in male and female rats was determined as 1948 and 2525 mg/kg bw/d, respectively, the highest dose tested (Endres et al., 2011). The test article contained 6.88×10^{10} CFU/g, therefore the NOAEL are equivalent to 1.34×10^{11} CFU/ kg bw/d and 1.74×10^{11} CFU/ kg bw/d, respectively.

Acute Skin Irritation

GanedenBC30™ (*B. coagulans* GBI-30, 6086) at a concentration of 1.93×10^{11} CFU/g, was tested for acute skin irritation in Male New Zealand White rabbits in accordance with OECD Guidelines for Testing of Chemicals No. 404; Acute Dermal Irritation/ Corrosion, adopted April 24, 2002. The test article was not classified as irritating to the skin according to EC directive 2001/59/EEC. The observed clinical sign of very slight erythema on the treated skin surface was concluded as fully reversible (Endres et al., 2009).

Acute Eye Irritation

GanedenBC30™ (*B. coagulans* GBI-30, 6086) at a concentration of 1.93×10^{11} CFU/g, was tested for acute eye irritation, in Male New Zealand White rabbits, in accordance with OECD Guidelines for Testing of Chemicals No. 405; Acute Eye Irritation/Corrosion, adopted April 24, 2002. The test article was not classified as irritating to the eye (Endres et al., 2009).

Reproductive Toxicity

This study was conducted in order to investigate the effects of GanedenBC30™ (*B. coagulans* GBI-30, 6086) administration on male and female reproductive performance in Wistar (HsdBrlHan) rats. This study was conducted in parallel to the one-year chronic toxicity study described above (Endres et al., 2011). Wistar rats were divided into four groups (males = 10/group; females = 20/group) and were fed a diet containing the *B. coagulans* preparation at a doses of 0, 600, 1,200 and 2,000 mg/kg bw/d. 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.

The mean doses of GanedenBC30™ in the various dose groups as analyzed from dietary intake were as follows: 600 mg/kg bw/d target dose group (males received a calculated mean daily intake of 715 mg/kg bw/d, females received 1,079 mg/kg bw/d); 1,200 mg/kg bw/d target dose group (males: 1,348 mg/kg bw/d, females: 2,082 mg/kg bw/d); 2,000 mg/kg bw/d target dose group (males: 2,372 mg/kg bw/d, females: 3,558 mg/kg bw/d). A further breakdown of the intake of GanednBC30™ in females of the parental generation showed the following mean intakes in the different treatment groups during the pre-mating, gestation and lactation periods: 600 mg/kg bw/d target group (908 mg/kg bw/d during pre-mating period, 775 mg/kg bw/d during gestation, 1,951 mg/kg bw/d during lactation); 1,200 mg/kg bw/d target group (1,769 mg/kg bw/d, 1,593 mg/kg bw/d, and 3700 mg/kg bw/d); 2,000 mg/kg bw/d target group (2,983 mg/kg bw/d, 2,596 mg/kg bw/d, and 6,438 mg/kg bw/d).

No mortality was reported in the parental generation. Pregnancy outcome, reproductive performance and live births were unaffected by treatment with GanedenBC30™. Necropsy showed hydrometra in 1/3 non-pregnant females from the control group and 2/5 in the 600 mg/kg bw/d group. No other alterations were found by necropsy. Histopathology revealed uterine dilation in 2/5 non-pregnant females in the 600 mg/kg bw/d group, 1/2 in the 1,200 mg/kg bw/d group and 1/1 in the 2,000 mg/kg bw/d group. No other alterations were reported following histopathological examination. There were no signs of treatment-related toxicity on the FO (parental) generation (male or female).

In terms of the offspring, there was a statistically significant slight increase in mortality in the 600 mg/kg bw/d treatment group compared to the control group. No treatment-related effects were reported in clinical observations, body weight gain and gross pathological examination in any of the offspring.

The authors concluded that GanedenBC30™ fed in the rat diet caused no signs of toxicity on the parental generation (male or female) of the same strain of rat during the course of the study. The No Observed Effect level (NOEL) for the parental group (reproductive performance) male and female rats was established as 2,372 and 3,558 mg/kg bw/d, respectively. The NOEL for the F1 offspring was determined as 3,558 mg/kg bw/d; the test article contained 6.88×10^{10} CFU/g, therefore the NOEL is equivalent to 2.45×10^{11} CFU/ kg bw/d (Endres et al., 2011).

Table 5. Genotoxicity Studies with *Bacillus coagulans*

***B. coagulans* Strain**	**Study Type**	**Strains or Species Tested**	**Metabolic Activation**	**Dose**	**Results**	**Reference**
GBI-30, 6086	Bacterial reverse mutation	*Salmonella typhimurium* TA98, 100, 1535, 1537, and *Escherichia coli* strain WP2[uvrA]	With and without S9 activation system	10, 50, 100, 500, and 5,000 µg per plate. Positive and negative controls performed as expected.	No mutagenic effect on any strain used in this test with and without metabolic activation.	Endres et al., 2009
GBI-30, 6086	Mouse micronucleus study	BALB/ dByJNarl mice (5 groups of 5)	No external activation system	500, 1,000, and 2,000 mg/kg bw/d of test article from a stock of 1.93×10^{11} CFU/g for 3 days. Positive and negative controls performed as expected.	No statistically significant increase in induction of micronuclei, ratio of reticulocytes to erythrocytes not significantly decreased compared to controls. No body weight differences, clinical signs, or toxicity in treated animals.	Endres et al., 2009
GBI-30, 6086	Chromosomal aberration assay	Chinese hamster ovary cells (CHO-K1 cells)	With and without S9 metabolic activation	According to OECD guidelines and using test stock material of 1.93×10^{11} CFU/g. Positive and negative controls performed as expected.	No statistically significant increase in chromosomal aberrations with or without metabolic activation.	Endres et al., 2009

Table 6. Pre-Clinical Studies with *Bacillus coagulans*

***B. coagulans* Strain**	**Study Type**	**Study Design (animal, # per group)**	**Duration**	**Dose**	**Results**	**Reference**
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 1.93×10^{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 hours. No corneal involvement or adverse signs in iris. Would not be classified as eye irritant.	Endres et al., 2009
GBI-30, 6086	Acute skin 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 1.93×10^{11} CFU/g moistened with water and applied to 6 cm^2 intact skin, then animals were wrapped.	Test results demonstrate that article is not irritating the skin. Slight erythema after 1h exposure, but all findings were minor and fully reversible.	Endres et al., 2009
Unique IS2	Acute toxicity	OECD Guideline 401, SD rats, 6/sex/dose	Single oral dose (gavage implied)	0, 3,250, and 6,500 mg/kg bw/d of test article of 10×10^9 CFU/g daily.	No treatment related effects were observed at any dose, including no findings of body weight changes, clinical signs, or gross pathological changes.	Sudha et al., 2011a
GBI-30, 6086	Acute oral toxicity	OECD Guideline 423, with 5 animals/sex/dose, Wistar Crl:(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 to remarkable differences between the treated and control animals.	Endres et al., 2009
CGMCC 9951	Short-term repeat dose toxicity	KM Mice, Females: n= 6/group; Males:	28 d	0, 1×10^6, 1×10^8, 1×10^{10} spores/kg bw/d	Authors conclude a safe dose of 1×10^{10} spores/kg bw/d	Gu et al., 2015

B. coagulans Strain	Study Type	Study Design (animal, # per group)	Duration	Dose	Results	Reference
		n = 5/group.				
Unique IS2	Short-term repeat dose toxicity	OECD Guideline 407, SD rats, 6 animals/sex/dose.	28 days	0, 130, 650, 1300 mg/kg bw/d, orally "fed" from stock test article of 5 x 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
GBI-30, 6086	Subchronic 13-week oral toxicity	OECD Guideline 408 as well as Red Book guidelines followed, with 10 animals/sex/dose, Wistar Crl:(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 x 10^{11} CFU/g.	NOAEL considered by authors to be >1000 mg/kg bw/d. 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.	Endres et al., 2009
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 x 10^{10} CFU/g.	• NOEL for parental male rats: 2372 mg/kg bw/d (mean value) • NOEL for parental female rats: 3558 mg/kg bw/d (mean value) • NOEL for reproductive performance of male rats: 2372 mg/kg bw/d (mean value) • NOEL for reproductive performance of female rats: 3558 mg/kg bw/d (mean value) • NOEL for F1 Offspring: 3558 mg/kg bw/d (mean value)	Endres et al., 2011

Unpublished Pre-Clinical Studies on the *B. coagulans* strain SANK 70258 Spores Preparation

Subchronic Oral Toxicity

Supporting information on the safety of the *B. coagulans* strain SANK 70258 has been generated under GLP conditions in a study sponsored by MKF (Ohnishi, 2015; Akagawa et al., 2016).[1] In an OECD (Guideline No. 408), GLP-compliant 90-day oral toxicity study, Sprague-Dawley rats (10 animals/sex/dose) were administered *B. coagulans* (Lot 141004AA, 5.09 x 10^{11} CFU/g, from which LACRIS-S is produced) by gavage, suspended in injection water only. Doses tested were 0, 500, 1000, and 2000 mg/kg bw/d with a dose volume of 10 mL/kg bw. Rats were observed daily for clinical signs of toxicity prior to the start of the study then twice daily during the dosing phase of the study. A functional observational battery (FOB) was performed on all animals once prior to the initiation of dosing, and once per week during the dosing phase of the study. Observations included approach response, touch response, auditory response, tail pinch response, aerial righting reaction and assessment of grip strength and motor activity. Body weights were measured and recorded prior to dosing and weekly thereafter. Food consumption was also recorded weekly and daily mean food consumption was calculated from total consumption over each period. Eye exams were performed prior to study start and at week 13. Urinalysis was also performed at week 13, as well as hematology and clinical chemistry. Appearance of major organs and weights of selected appropriate organs (heart, thymus, spleen, lungs, liver, kidney, testes, epididymis, prostate, pituitary, thyroids, submandibular glands, adrenals, brain, ovary, uterus) were recorded at necropsy. Although the exact organs examined by histopathology were not listed in the publication, since the study was conducted per OECD guidelines, all major organs and any gross lesions in the control and high dose groups would have been examined.

There were no dead or moribund animals during the study period, and no treatment-related clinical changes were reported. There were also no treatment-related alterations in the results of the FOB, grip strength or motor activity assessments. Treatment likewise did not affect body weight or food consumption. The authors reported statistically significant lower mean corpuscular hemoglobin concentration (MCHC) in males at the 1000 mg/kg bw/d dose and higher values of monocyte ratio and/or count in males at the 500 and 1000 mg/kg bw/d doses and in females at the 1000 mg/kg bw/d dose when compared to the control group. However, a dose-response relationship was not present, indicating that the effect was incidental and not related to treatment. Similarly, statistically significant shortening of partial thromboplastin and activated partial thromboplastin time was observed in males at the 500 mg/kg bw/d dose and higher when compared to the control group. However, these values were within the range of historical control data for the laboratory, indicating that they were incidental physiological fluctuations and not treatment-related. Total protein and albumin in males at the 1000 mg/kg bw/d dose and total bilirubin in females at the 1000 mg/kg bw/d dose were statistically significantly decreased as well, when compared to the control group. However, the effect lacked dose-response. The only other alteration was significantly higher aspartate transaminase levels in males at the highest dose tested (2000 mg/kg bw/d) compared to the control group, but the authors stated that all such differences in clinical chemistry were sporadic and within historical controls ranges for the

[1] The 90-day toxicity study of *B. coagulans* strain SANK 70258 was unpublished when the GRAS Expert Panel convened (Ohnishi, 2015), but has since been published in the scientific literature (Akagawa et al., 2016).

testing facility. Thyroid weight was statistically significantly increased in males at the highest dose tested (absolute weight only, relative not calculated in study report), when compared to the control group, but the organ weight increase lacked corresponding histopathology and was thus considered to be incidental. Likewise, minor changes noted at necropsy and in the histopathology were incidental and not treatment-related.

The no observable adverse effect level (NOAEL) for the study was 2000 mg/kg bw/d, the highest dose tested, in males and females. Because dosing was via gavage, this represents the exact, body weight adjusted dose delivered. Utilizing the spore concentration value of 5.09×10^{11} CFU/g for the *B. coagulans* test material in this study, the study NOAEL is equivalent to a daily intake of 1.02×10^{12} CFU/kg bw/d (2 g/kg bw/d x 5.09×10^{11} CFU/g) in male and female rats. Applying an uncertainty factor of 10x for interspecies variability, 10x for intraspecies variability, and 10x for the extrapolation of a subchronic duration study to chronic duration intake, a composite uncertainty factor of 1000x is derived. Applying this uncertainty factor to the NOAEL of 1.02×10^{12} CFU/kg bw/d results in an acceptable daily intake (ADI) of 1.02×10^{9} CFU/kg bw/d for *B. coagulans* of the strain relevant to LACRIS-S preparations.

Oral Feeding Studies

In support of a feed additive petition for LACRIS-S in Japan, unpublished, internal data were submitted to the Japanese authorities (Yamaguchi et al., 1969; Maegami, 1969; Igarashi, 1969; Department of Stockbreeding Chemistry, 1969; Iwado et al., 1966). The strain of the *B. coagulans* test material used in these studies is assumed to be SANK 70258. Limited information is available from these studies to evaluate safety of the preparation for human consumption though they are included in this review for completeness.

The effect of LACRIS-S (containing *B. coagulans* SANK 70258) on growth promotion and prevention or treatment of diarrhea in piglets and calves has been examined by several investigators. Some of these studies provided the dose in CFU/kg feed, but did not provide the daily feed intake and body weights of the piglets. Overall, the data suggest that LACRIS at doses of 10^{7} to 3×10^{7} CFU/kg bw/d for 3 days to 7 weeks (49 days) did not have any deleterious effect on growth. If we assume that the body weight of piglets is approximately 10 kg and the average consumption amount is approximately 1 kg/d, dietary intake of *B. coagulans* is roughly10^{8} CFU/d for piglets. No other major safety parameters were observed and reported in these studies.

Pre-Clinical Safety Summary

Several strains of *B. coagulans* have been examined in a variety of pre-clinical studies. The *B. coagulans* strains tested in pre-clinical studies are very similar to *B. coagulans* SANK 70258 and these data can be used to support the safety of *B. coagulans* SANK 70258 in a spores preparation. Studies of several strains of *B. coagulans* indicate that the organism is not acutely toxic or irritating, is not genotoxic, and is not a reproductive toxin. The longest duration oral toxicity study of *B. coagulans* was a one-year chronic toxicity study of *B. coagulans* GBI-30, 6086 (Endres et al., 2011). The lowest NOAEL from all groups in this study was 1948 mg/kg bw/d of the test article. The test article was reported in Endres et al., 2011, to have spore content of 6.88×10^{10} CFU/g, resulting in a NOAEL of 1.34×10^{11} CFU/kg bw/d (1.948 g/kg bw/d x

6.88×10^{10} CFU/g). Applying an uncertainty factor of 10x each for intra- and inter-species variability to this chronic NOAEL results in an ADI of 1.34×10^9 CFU/kg bw/d. This ADI was corroborated by the ADI derived from the unpublished 90-day study of the same strain of *B. coagulans* contained in LACRIS-S preparation, strain SANK 70258, which was 1.02×10^9 CFU/kg bw/d based on the highest dose tested (Ohnishi, 2015) (Note: this toxicity study was published after the GRAS conclusion was reached (Akagawa et al., 2016)). At 1.36×10^8 CFU/kg bw/d, the ADI derived from the 90-day study of *B. coagulans* GBI-30, 6086 (Endres et al., 2009) is an order of magnitude lower than these ADI values, but the NOAEL from the subchronic study by Endres et al., (2009) was the highest dose tested, suggesting that the true NOAEL is likely higher. The relevant ADI for the present safety assessment is based upon that which is derived from the chronic study of *B. coagulans* GBI-30, 6086. Endres and colleagues calculated an ADI of 1.34×10^9 CFU/kg bw/d or 93.8×10^9 CFU/d assuming a 70 kg bw.

Clinical Data

Fate of B. *coagulans* in the Human Gastrointestinal Tract

B. coagulans is a spore-forming bacteria. Following consumption, the spores pass through the acid environment of the stomach and reach the duodenum where they germinate and rapidly multiply (Losada and Olleros, 2002). As summarized in GRN 526 (Unique Biotech Ltd., 2014), passage into the duodenum occurs approximately 4 hours after ingestion, and lactic acid production begins following germination in the duodenum. Presence of *B. coagulans* in the intestinal tract is transient as the spores are excreted over an approximately one (1) week period following ingestion.

B. coagulans SANK 70258

Five clinical studies of *B. coagulans* SANK 70258 supplementation as reported in four publications were identified (Ara et al., 2002; Iino et al., 1997a; Iino et al., 1997b; Kajimoto et al., 2005). The spores preparation was administered to healthy subjects, individuals with allergies, and individuals tending to experience constipation. Study details are provided in Table 7 and summarized below.

In a randomized, double-blind, placebo-controlled clinical trial, Kajimoto and colleagues (2005) studied the effects of *B. coagulans* SANK 70258 on seasonal allergic rhinitis. Fifty-eight healthy volunteers, aged 20-65 years, with a history of Japanese cedar pollinosis were enrolled in the study (55 completed the study). Subjects were randomized to receive test food containing *B. coagulans* SANK, at a dose of 4×10^8, or placebo for 8 weeks. No significant changes in 9 common hematology parameters or 25 common clinical chemistry parameters were noted and no adverse reactions were reported in subjects receiving *B. coagulans*.

In an uncontrolled intervention, Ara and colleagues (2002) examined the effects of *B. coagulans* SANK 70258 on the intestinal environment in healthy adults. Twenty healthy adults, added 20 to 40 years were enrolled in the study. Study participants were given 1×10^8 CFU dried *B. coagulans* SANK 70258 powder daily for 2 weeks. No reports of adverse effects or intolerance of the supplements were noted while consuming the powder. Ara and colleagues conducted a second intervention to examine effects of *B. coagulans* on dermal characteristics. In this study,

23 females, aged 20 to 40 years, with a propensity for constipation were given 1 x 10^8 CFU dried *B. coagulans* SANK 70258 powder daily for 4 weeks. No reports of adverse effects or intolerance of the supplements were noted.

In a trial of 28 healthy female adults, Iino and colleagues (1997a) examined the effect of *B. coagulans* on various properties of feces. Study subjects were randomized to receive dried powder of *B. coagulans* SANK 70258 providing 1.8 x 10^8 CFU per day for 2 weeks. No tolerance data were reported and there were no reports of adverse events. To determine the ability of *B. coagulans* to improve intestinal bacterial flora, Iino and colleagues (1997b) conducted a second randomized trial in 18 healthy female adults. The women were randomized to receive the spore-forming lactic acid bacteria *B. coagulans* SANK 70258 at a dose of 2 x 10^7 CFU per day, 1 x 10^8 CFU per day, or 2 x 10^8 CFU per day for 2 weeks. None of the subject complained of gas, diarrhea, or abdominal pain due to *B. coagulans*.

Losada and Olleros (2002) also reported findings of seven short clinical trials (up to 20 days) conducted with Lacbon tablets providing up to 6 x 10^8 CFU daily (presumably strain SANK 70258) as summarized by the Sankyo Co. Ltd., in Japan; untoward effects were not noted in this summary.

Table 7. Clinical Trials with *Bacillus coagulans* SANK 70258

Reference	Study Type	Study Population (population, age and sex, # allocated [completed])	Duration of Intake	CFU/d	Adverse Effects
Kajimoto et al., 2005	RCT 2 groups	Subjects with seasonal allergic rhinitis 20-65 y, 38.5 ± 7.6 y (treatment), 38.1 ± 7.2 y (control); M/F n = 58 [n = 29 treatment, n = 26 control]	8 wk	0.4×10^9	No significant changes in hematology and clinical chemistry parameters noted. No adverse reactions reported in subjects receiving *B. coagulans*
Ara et al., 2002	Uncontrolled intervention	Healthy subjects 20-40 y; M/F n = 20	2 wk	0.1×10^9	No mention of adverse effects
Ara et al., 2002	Uncontrolled intervention	Volunteers with tendency for constipation 20-40 y; F n = 23	4 wk	0.1×10^9	No mention of adverse effects
Iino et al., 1997a	RT 2 groups	Healthy women age not reported; F n = 28	2 wk	0.1×10^9	No mention of adverse effects
Iino et al., 1997b	RT 3 groups	Healthy women age not reported; F n = 18	2 wk	0.02×10^9 0.1×10^9 0.2×10^9	No subject complained of gas, diarrhea, or abdominal pain due to ingestion of *B. coagulans*

Abbreviations: CFU – colony forming units; F – female; M – male; RCT – randomized controlled trial; RT – randomized trial; wk – weeks; y - years

Other Strains

Other strains of *B. coagulans* have been the subject of numerous clinical trials, and results from these trials provide additional information on the tolerability and suitability of *B. coagulans* for human consumption (see Table 8). Twenty-three additional clinical trials were identified in safety reviews of *B. coagulans* previously conducted to support GRAS determinations and the reviews of PubMed for publications since 2013. Extracted information includes details on study design, study population (description, age, sex, number enrolled), study duration (duration of supplementation), and CFU/d. Although the studies were not designed specifically to assess safety, reported information on adverse events, clinical chemistries, tolerance, or other observations relevant for safety were noted. Studies were conducted in diverse populations including healthy and preterm infants, children with abdominal pain, healthy adults, and adults with various conditions including hyperlipidemia, type 2 diabetes, arthritis, HIV, irritable bowel syndrome (IBS) and other gastrointestinal disorders. Daily intake of *B. coagulans* ranged from 0.03 to approximately 20 x 10^9 CFU per day for periods of 3 days to 1 year.

Summary of Clinical Data

Results from four publications detailing clinical trials with *B. coagulans* SANK 70258 and more than 20 published clinical trials with other strains of *B. coagulans* and species of unspecified strain provide evidence that daily intake of *B. coagulans* occurred with no reports of serious adverse effects or observed safety concerns. Daily intake of *B. coagulans* was up to approximately 20 x 10^9 CFU. The longest duration of *B. coagulans* supplementation, a study of infants receiving 0.1 x 10^9 CFU/d, spanned a period of 1 year (Chandra 2002). In several studies in adults, the period of intervention was in the range of 8 to approximately 13 weeks (Sudha et al., 2012a; Sudha et al., 2011b; Yang et al., 2014; Mandel et al., 2010; Dolin et al., 2009; Hun et al., 2009; Rogha et al., 2014; Majeed et al., 2016; Mohan et al., 1990a,b). Reports of mild to moderate gastrointestinal symptoms with intake of *B. coagulans* were noted in some studies, though the effects were generally self-limiting and reversible. Overall, results from these studies in both healthy and compromised individuals indicate that under the tested conditions of use, *B. coagulans* presented no evidence of pathogenicity or toxicity and was generally well tolerated.

Table 8. Clinical Trials with *Bacillus coagulans* Strains Not Identified as *B. coagulans* SANK 70258

Reference	*B. coagulans* Strain	Study Type	Study Population (population, age and sex, # randomized [completed])	Duration of intake	CFU/d	Findings
***B. coagulans* Unique IS-2 (MTCC-5260)**						
Saneian et al., 2015	*B. coagulans* Unique IS-2 + FOS	RCT 2 groups	Children with functional abdominal pain 6-18 y, 9.0 ± 2.2 y (treatment), 8.5 ± 2.2 y (control); M/F n = 59 [45] treatment, n = 56 [43] control	4 wk	0.3×10^9 FOS – 200 mg/d	No serious adverse effects of *B. coagulans* were found. 5 patients (8%) had to stop medication due to side effects. Observed side effects were comparable between the treatment and control groups.
Sudha et al., 2011b	*B. coagulans* Unique IS-2 (MTCC-5260)	Open label 3 groups	Men and women with hyperlipidemia 42-53 y; M/F n = 10/group	60 d	10×10^9 20×10^9	No mention of adverse effects
Sudha et al., 2012a	*B. coagulans* 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 d	4×10^9	No mention of adverse effects
Sudha et al., 2012b	*B. coagulans* 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 d	4×10^9	Significant reductions in counts of RBC and WBC and serum creatinine levels was observed however values were within the normal range No other changes in safety parameters were observed.
***B. coagulans* GIB-30, 6086**						
Nyangale et al., 2015	*B. coagulans* GIB-30, 6086	Crossover 2 groups	Healthy men and women 65-80 y; M/F n = 42 [36]	28 d	1×10^9	No mention of adverse effects
Yang et al., 2014	*B. coagulans* GIB-30, 6086	RCT 2 groups	HIV infected persons receiving cART Median age 49 y (treatment), 51 y	90 d	2×10^9	No serious adverse events were reported. Only mild gastrointestinal symptoms were reported during the study; in the probiotic

Reference	*B. coagulans* Strain	Study Type	Study Population (population, age and sex, # randomized [completed])	Duration of intake	CFU/d	Findings
			(control); M/F n = 12 [10] treatment, n = 12 [7] control			group, 3/10 reported bloating. In the placebo group, 1/7 reported increased diarrhea.
Kimmel et al., 2010	*B. coagulans* GBI-30, 6086	Open label 1 group	Healthy subjects 27 y; M/F n = 10 (10)	28 d	0.5×10^9	No serious adverse events were reported throughout the study
Mandel et al., 2010	*B. coagulans* GBI-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 d	2×10^9	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.
Baron et al., 2009	*B. coagulans* GBI-30, 6086	Crossover 2 groups	Healthy adults 44 y; M/F n = 10 [9]	30 d	2×10^9	No serious adverse events were reported throughout the study.
Dolin et al., 2009	*B. coagulans* GBI-30, 6086	RCT 2 groups	Patients with diarrhea prominent IBS 52.3 ± 11.1 y (treatment), 44.0 ± 17.9 y (control); M/F n = 26 [26] treatment, n = 29 [26]placebo	8 wk	2×10^9	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.

Reference	*B. coagulans* Strain	Study Type	Study Population (population, age and sex, # randomized [completed])	Duration of intake	CFU/d	Findings
Hun et al., 2009	*B. coagulans* GBI-30, 6086	RCT 2 groups	IBS-abdominal pain and bloating patients 48.36 y; M/F n = 50 [22/group]	8 wk	~0.8 x 10^9	No treatment related adverse events or serious adverse events reported during the study period. Four adverse events reported in the placebo group and 2 in the treatment group were unrelated to the treatments.
Kalman et al., 2009	*B. coagulans* GBI-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 wk	2 x 10^9	"…the *Bacillus coagulans*-based probiotic product was effective and safe..."
***B. coagulans* MTCC 5856**						
Majeed et al., 2016	*B. coagulans* 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 d	2 x 10^9	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. 5 dropouts (1 treatment, 4 control) due to personal reasons. Significantly reduced discomfort (bloating, vomiting, diarrhea, stool frequency, abdominal pain) with treatment
Strain not specified						
Asemi et al., 2015	*B. coagulans*[a] + inulin + *B*-carotene	Crossover 2 groups	Patients with type 2 diabetes 52.9 ± 8.1 y; M/F n = 51 [48]	6 wk	0.03 x 10^9	No serious adverse reactions were reported

Reference	*B. coagulans* Strain	Study Type	Study Population (population, age and sex, # randomized [completed])	Duration of intake	CFU/d	Findings
Asgarshir-azi et al., 2015	*B. coagulans* + FOS	RCT 3 groups	Children with chronic abdominal pain (> 2 mo with recurrence at least once per week) 7.44 ± 2.44 y (*B coagulans* + FOS), 7.06 ± 2.38 y (peppermint oil), 7.42 ± 2.49 y (control); M/F n = 40 [29] *B. coagulans* + FOS; n = 40 [34] peppermint oil; n = 40 [25] control	1 mo	0.5×10^9	No adverse reactions or intolerance observed
Bahmani et al., 2015; Tajadadi-Ebrahimi et al., 2014; Shakeri et al., 2014	*B. coagulans*[a]	RCT 3 groups	Diabetic patients 51.3 ± 10.4 y (synbiotic), 52.0 ± 7.2 y (probiotic), 53.4 ± 7.5 y (control); M/F (81% F) n = 27 [25] synbiotic, n = 27 [25] probiotic, n = 27 [26] control	8 wk	0.3×10^9	No side effects were reported following the consumption of the probiotic bread
Chandra, 2002	*B. coagulans*[a]	RCT 2 groups	Full-term healthy newborn infants newborns; M/F n = 55 treatment, n = 57 control	1 y	0.1×10^9	No mention of adverse effects
Cui et al., 2004	*B. coagulans*	RCT 2 groups	Patients with acute or chronic diarrhea 18-65 y; M/F n = 103 treatment, n = 101 control	3-7 d (acute diarrhea) 14-21 d (chronic diarrhea)	0.3×10^9	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.
Dutta et al., 2011	*B. coagulans*	RCT 2 groups	Children with acute watery diarrhea and 'some' dehydration 12 ± 4 y (treatment), 11 ± 4 y (control); M/F n = 80 [78] treatment, n = 80 [70] control	5 d	0.24×10^9	No adverse event or complication was observed

Reference	*B. coagulans* Strain	Study Type	Study Population (population, age and sex, # randomized [completed])	Duration of intake	CFU/d	Findings
Minamida et al., 2015	*B. coagulans* 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 wk	0.1×10^9	No mention of adverse effects
Mohan et al., 1990a, b	*B. coagulans*[a]	Open label 1 group	Patients with primary hyperlipidemia 45.6 y; M/F; n = 20 [17]	12 wk	0.36×10^9	No adverse effects were noted except constipation in one patient
Rogha et al., 2014	*B. coagulans* + 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 wk	0.45×10^9 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 specific side effects were observed.
Sari et al., 2011	*B. coagulans*[a]	RCT 2 groups	Preterm infants with a gestational age of < 33 wk or birth weight of < 1500 g 2 d; M/F n = 121 [110] treatment, n = 121 [111] control	34.5 or 30 d (median)	0.35×10^9	The incidence of sepsis did not significantly differ between groups Other adverse effects attributed to *L. sporogenes* (flatulence, diarrhea) were not observed

[a] Referred to 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; wk – weeks; y - years

Conclusion Regarding Safety and General Recognition of Safety

The safety of the proposed use of *B. coagulans* SANK 70258 in a spores preparation (up to 2 x 10^9 CFU/serving) includes use as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups. The estimated daily intake of *B. coagulans* SANK 70258 from these uses is 36.4 x 10^9 CFU per day.

The available evidence using conventional phenotypic analysis in combination with genotypic analysis confirms the identity of the *B. coagulans* SANK 70258 microorganism as a strain of *B. coagulans*. *B. coagulans* is classified as BSL-1 organism, indicating the organism is not known to cause disease in healthy human adults. Published studies of several strains of *B. coagulans* indicate that the organism is not acutely toxic or irritating, is not genotoxic, and is not a reproductive toxin. The available evidence also shows that *B. coagulans* is not resistant to many common antibiotics and the strain is not known to have virulence activity. Unpublished pre-clinical studies of *B. coagulans* SANK 70258 corroborate these findings. Published clinical studies in healthy and compromised individuals indicate that use of *B. coagulans* presented no evidence of pathogenicity or toxicity and was generally well tolerated; daily intake of *B. coagulans* was up to approximately 20 x 10^9 CFU. The longest duration of *B. coagulans* supplementation, a study of infants receiving 0.1 x 10^9 CFU/d, spanned a period of 1 year. In several studies in adults, the period of intervention was in the range of 8 to approximately 13 weeks. The analytical data on *B. coagulans* and pre-clinical and clinical studies are supported by decades of use with no reported adverse effects. It is therefore reasonable to conclude that the proposed use of *B. coagulans* SANK 70258 in a spores preparation is safe within the meaning of 21 CFR 170.3(i), i.e., meets the standard of reasonable certainty of no harm.

General recognition of safety requires common knowledge throughout the scientific community knowledgeable about the safety of food ingredients that there is a reasonable certainty that a substance is not harmful under the conditions of its intended use in foods. The regulatory and scientific reviews needed to establish the safety of the intended use of *B. coagulans* SANK 70258 in a spores preparation are published in the scientific literature and, therefore, are generally available to the community of qualified food ingredient safety experts. There is broad-based and widely disseminated knowledge concerning *B. coagulans*. The publicly available data and information supporting the safety of the intended use of *B. coagulans* SANK 70258 in a spores preparation in foods as specified in this document are not only generally available, but are also generally accepted among qualified food safety experts. The proposed use of *B. coagulans* SANK 70258 in a spores preparation in select foods therefore can be concluded to be safe and generally recognized as safe (GRAS) through scientific procedures.

An independent panel of scientific experts, qualified by training and experience to evaluate the safety of food ingredients, has critically reviewed and evaluated the publicly available information summarized in this document. They have individually and collectively concluded

that *B. coagulans* SANK 70258 in a spores preparation produced consistent with Good Manufacturing Practice and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further unanimously concludes that these uses of *B. coagulans* SANK 70258 in a spores preparation are GRAS based on scientific procedures, and that other experts qualified to assess the safety of foods and food ingredients would concur with these conclusions. The Panel's GRAS opinion is included as **Error! Reference source not found.** to this document.

Discussion of Information Inconsistent with GRAS Determination

No information has been identified that would be inconsistent with a finding that the proposed use of *B. coagulans* SANK 70258, meeting appropriate specifications specified herein and used according to Good Manufacturing Practice (GMP), is GRAS.

Part 7. List of Supporting Data and Information in GRAS Notice

Akagawa Y, Ohnishi Y, Takaya M, Watanabe Y. Safety assessment of probiotic bacteria, Bacillus coagulans strain SANK70258, in rats. Fundam. Toxicol. Sci. 2015;3(6):243-250.

Ara K, Meguro S, Hase T, Tosimitsu I, Otsuji K, Kawai S, Ito S, Iiino H. Effect of spore-bearing lactic acid-forming bacteria (*Bacillus coagulans* SANK 70258) administration of the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics in humans and rats. Microbial Ecol. Health Disease. 2002;14:4-13.

Asemi Z, Alizadeh SA, Ahmad K, Goli M, Esmaillzadeh A. 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. 2015. pii: S0261-5614(15)00186-7.

Asgarshirazi M, Shariat M, Dalili H. 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. 2015;17(4):e23844.

Bahmani F, Tajadadi-Ebrahimi M, Kolahdooz F, Mazouchi M, Hadaegh H, Jamal AS, Mazroii N, Asemi S, Asemi Z. 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. 2015:1-8.

Banerjee C, Bustamante CI, Wharton R, Talley E, Wade JC. Bacillus infections in patients with cancer. Arch. Intern. Med. 1998;148:1769-1774.

Baron M. A patented strain of *Bacillus coagulans* increased immune response to viral challenge. Postgrad Med. 2009;121(2):114-8.

Chandra RK. Effect of Lactobacillus on the incidence and severity of acute rotavirus diarrhea in infants. A prospective placebo-controlled double-blind study. Nutrition Research. 2002;22:65-9.

Codex Alimentarius Commission (CAC). List of CODEX specifications for food additives. Available at: www.codexalimentarius.org/input/download/standards/9/CXA_006s.pdf. Accessed April, 2015.

Cui Y, Wan FC, Tang DL, Wu SH. Efficacy of *Bacillus coagulans* tablets in the treatment of acute and chronic diarrhea. Int J Immunotherapy. 2004;20:17-22.

Dolin BJ. Effects of a proprietary *Bacillus coagulans* preparation on symptoms of diarrhea-predominant irritable bowel syndrome. Methods Find Exp Clin Pharmacol. 2009;31(10):655-9.

Dutta P, Mitra U, Dutta S, Rajendran K, Saha TK, Chatterjee MK. 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. 2011;16(5):555-61.

Endres JR, Clewell A, Jade KA, Farber T, Hauswirth J, Schauss AG. Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient. Food Chem Toxicol. 2009;47(6):1231-8.

Endres JR, Qureshi I, Farber T, Hauswirth J, Hirka G, Pasics I, Schauss AG. One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. Food Chem Toxicol. 2011;49(5):1174-82.

European Food Safety Authority (EFSA). Scientific Opinion of the Panel on Biological Hazards on a request from EFSA on the maintenance of the QPS list of microorganisms intentionally added to food or feed. The EFSA Journal; 923, 1-48.

European Food Safety Authority BIOHAZ Panel (EFSA Panel on Biological Hazards), 2015. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. 2: Suitability of taxonomic units notified to EFSA until March 2015. EFSA Journal 2015;13(6):4138, 29 pp. doi:10.2903/j.efsa.2015.4138.

Food Standards Australia New Zealand (FSANZ). Novel Food Record of views. 2015. Available at http://www.foodstandards.gov.au/industry/novel/novelrecs/pages/default.aspx.

Gu SB, Zhao LN, Wu Y, Li SC, Sun JR, Huang JF, Li DD. Potential probiotic attributes of a new strain of *Bacillus coagulans* CGMCC 9951 isolated from healthy piglet feces. World J Microbiol Biotechnol. 2015;31(6):851-63.

Hammer, BW 1915. Bacteriological studies on the coagulation of evaporated milk. Iowa Agric. Exp. Stn. Res. Bull. 19:119-131.

Health Canada. 2015. Health Canada's List of Permitted Food Enzymes (Lists of Permitted Food Additives). Available at: http://www.hc-sc.gc.ca/fn-an/securit/addit/list/5-enzymes-eng.php. Accessed April 2015.

Hun L. *Bacillus coagulans* significantly improved abdominal pain and bloating in patients with IBS. Postgrad Med. 2009;121(2):119-24.

Igarashi T. Experiment on the growth-promoting effect of "Lacris-10 for animals" on pigs. 1969:21-22. Unpublished.

Iino H, Matsumoto Y, Ito M, Abe S, Sugara T. Research on the fecal improvement effect due to lactic acid forming bacillus bacteria (*Bacillus coagulans*). Prog Med. 1997a;17:3299-3303 (Article in Japanese; English translation provided by MKF).

Iino H, Matsumoto Y, Ito M, Abe S, Sugara T. Research on the human intestinal flora improvement effect by lactic acid forming bacillus bacteria (*Bacillus coagulans*). Prog Med. 1997b;17:3303-08 (Article in Japanese; English translation provided by MKF).

Irwin S. Comprehensive observational assessment: In. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia. 1968;13(3):222-57.

Isu NR, Njoku HO. An evaluation of the microflora associated with fermented African oil bean (Pentaclethra macrophylla Bentham) seeds during ugba production. Plant Foods Hum Nutr. 1997;51(2):145-57.

Iwado S, Otake Y, Watari M. Study of Lacris bacteria (14th report). Lacris bacteria on E. coli in feces of piglets given feed supplemented with Lacris bacteria. Unpublished report by Central Research Laboratories, Sankyo Co., Ltd. 1966. Unpublished.

Kajimoto O, Suwa M, Takahashi Y, Uchida N, Hayama M, Tamura M, Nakagawa S, Kajimoto Y. Effect of *Bacillus coagulans* SANK70258 on seasonal allergic rhinitis (Japanese Cedar Pollinosis): A randomized, placebo controlled, double-blind study. J New Rem Clin. 2005;54(11):107-25 (Article in Japanese; English translation provided by MKF).

Kalman DS, Schwartz HI, Alvarez P, Feldman S, Pezzullo JC, Krieger DR. 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. 2009;9:85.

Khatri I, Sharma S, Ramya TN, Subramanian S. Complete Genomes of *Bacillus coagulans* S-lac and Bacillus subtilis TO-A JPC, Two Phylogenetically Distinct Probiotics. PLoS One. 2016 ;11(6):e0156745. doi:10.1371/journal.pone.0156745. eCollection 2016.

Kimmel M, Keller D, Farmer S, Warrino DE. A controlled clinical trial to evaluate the effect of Ganeden BC(30) on immunological markers. Methods Find Exp Clin Pharmacol. 2010;32(2):129-32.

Kodama, K. Identification of LACRIS-S bacteria. Tsukuba Research Laboratories, Sankyo Co., Ltd. June, 1992. Unpublished.

Krawczyk AO, de Jong A, Holsappel S, Eijlander RT, van Heel A, Berendsen EM, Wells-Bennik MH, Kuipers OP. Genome Sequences of 12 Spore-Forming Bacillus Species, Comprising *Bacillus coagulans, Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus sporothermodurans*, and *Bacillus vallismortis*, Isolated from Foods. Genome Announc. 2016;4(3). pii: e00103-16. doi: 10.1128/genomeA.00103-16.

Logan, NA, Hoffmaster AR, Shadomy SV, Stauffer KE. Bacillus and other Aerobic Endospore-forming Bacteria. In Clinical Microbiology 10th Edition. Versalovic, Carroll JKC, Funke G, Jorgensen JH, Landry ML, and Warnock DW(Eds.). ASM Press, Washington DC. 2011.

Losada MA, Olleros T. Towards a healthier diet for the colon: the influence of fructooligosaccharides and lactobacilli on intestinal health. Nutrition Research. 2002;22:71-84.

Maegami M. About the growth-promoting effect of Lacris on pigs. Mitsubishi-Kagaku Foods Internal Study. 1969:15-18. Unpublished.

Majeed M, Nagabhushanam K, Natarajan S, Sivakumar A, Ali F, Pande A, Majeed S, Karri SK. *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. 2016;15:21.

Majeed M, Nagabhushanam K, Natarajan S, Sivakumar A, Eshuis-de Ruiter T, Booij-Veurink J, de Vries YP, Ali F. Evaluation of genetic and phenotypic consistency of *Bacillus coagulans* MTCC 5856: a commercial probiotic strain. World J Microbiol Biotechnol. 2016;32(4):60. doi: 10.1007/s11274-016-2027-2. Epub 2016 Feb 29.

Mandel DR, Eichas K, Holmes J. *Bacillus coagulans:* a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. BMC Complement Altern Med. 2010;10:1.

Minamida K, Nishimura M, Miwa K, Nishihira J. Effects of dietary fiber with *Bacillus coagulans* lilac-01 on bowel movement and fecal properties of healthy volunteers with a tendency for constipation. Biosci Biotechnol Biochem. 2015;79(2):300-6.

Mohan JC, Arora R, Khalilullah M. Preliminary observations on effect of *Lactobacillus sporogenes* on serum lipid levels in hypercholesterolemic patients. Indian J Med Res. 1990a;92:431-2.

Mohan JC, Arora R, Khalilullah M. Short term hypolipidemic effects of oral lactobacillus sporogenes therapy in patients with primary dyslipidemias. Indian Heart J. 1990b Sep-;42(5):361-4.

Moldenhauer JE, Bass S, Rubio SL. Antimicrobial resistance of three species of Bacillus to thirty various antimicrobial agents. PDA J Pharm Sci Technol. 1996 ;50(5):324-5.

Nakayama O and Yano M. 1951. Nippon Nogeikagaku Kaishi. 117-120. Unpublished.

NBRC. Catalogue detail information. NBRC 12583. http://www.nbrc.nite.go.jp/NBRC2/NBRCCatalogueDetailServlet?ID=NBRC&CAT=00012583. Accessed June 23, 2014.

Nyangale EP, Farmer S, Cash HA, Keller D, Chernoff D, Gibson GR. *Bacillus coagulans* GBI-30, 6086 Modulates Faecalibacterium prausnitzii in Older Men and Women. J Nutr. 2015;145(7):1446-52.

Ohnishi Y. A 90-day oral gavage repeated-dose toxicity study of *Bacillus coagulans* in rats; Study No. B150161. November 27, 2015. LSI Medience Corporation. Unpublished.

Pariza MW, Gillies KO, Kraak-Ripple SF, Leyer G, Smith AB. Determining the safety of microbial cultures for consumption by humans and animals. Regul Toxicol Pharmacol. 2015;73(1):164-71.

Reed, R, EGD. Murray, and NR Smith. Bergey's Manual of Determinative Bacteriology. Seventh Edition. 1957. The Williams and Wilkins Company, Baltimore.

Rogha M, Esfahani MZ, Zargarzadeh AH. The efficacy of a synbiotic containing *Bacillus Coagulans* in treatment of irritable bowel syndrome: a randomized placebo-controlled trial. Gastroenterol Hepatol Bed Bench. 2014;7(3):156-63.

Sabinsa Corporation. 2015. GRAS Notice: *Bacillus coagulans* SBC37-01 spores preparation. (GRN 601), available at http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=601&sort=GRN_No&order=DESC&startrow=1&type=basic&search=bacillus%20coagulans.

Sabinsa Corporation. 2016. Sabinsa Receives "No Questions" Letter from US FDA on GRAS Status for Lactospore®. http://www.sabinsa.com/newsroom/press-releases/pr20160503.html; Accessed July 6, 2016.

Sakuma R. Hashima Laboratory, Nihon Bioresearch Inc. Antibiotic Susceptibility Test of *Bacillus Coagulans* SANK70258 Spore Preparation (Product Name: LACRIS-S). Study No. 360021. 2016. Final Report (3rd Draft), June 30, 2016. Unpublished.

Salvetti E, Orrù L, Capozzi V, Martina A, Lamontanara A, Keller D, Cash H, Felis GE, Cattivelli L, Torriani S, Spano G. Integrate genome-based assessment of safety for probiotic strains: *Bacillus coagulans* GBI-30, 6086 as a case study. Appl Microbiol Biotechnol. 2016;100(10):4595-605.

Sanders ME, Morelli L, Tompkins TA. Sporeformers as human probiotics: *Bacillus, Sporolactobacillus*, and *Brevibacillus*. Comprehensive Reviews in Food Science and Food Safety. 2003;2:100-10.

Saneian H, Pourmoghaddas Z, Roohafza H, Gholamrezaei A. Synbiotic containing *Bacillus coagulans* and fructo-oligosaccharides for functional abdominal pain in children. Gastroenterol Hepatol Bed Bench. 2015;8(1):56-65.

Sanzyme Limited. 2015. GRAS Notice: *Bacillus coagulans* SNZ1969 spores preparation. (GRN 597), available at

http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=597&sort=GRN_No&order=DESC&startrow=1&type=basic&search=bacillus%20coagulans.

Sari FN, Dizdar EA, Oguz S, Erdeve O, Uras N, Dilmen U. Oral probiotics: Lactobacillus sporogenes for prevention of necrotizing enterocolitis in very low-birth weight infants: a randomized, controlled trial. Eur J Clin Nutr. 2011;65(4):434-9.

Shakeri H, Hadaegh H, Abedi F, Tajabadi-Ebrahimi M, Mazroii N, Ghandi Y, Asemi Z. Consumption of synbiotic bread decreases triacylglycerol and VLDL levels while increasing HDL levels in serum from patients with type-2 diabetes. Lipids. 2014;49(7):695-701.

Sudha RM, Bhonagiri S. Efficacy of *Bacillus coagulans* strain IS-2 in the treatment of patients with acute diarrhea. International Journal of Probiotics and Prebiotics. 2012b;7(1):33-37.

Sudha RM, Chauhan P, Dixit K, Babu S, Jamil K. Molecular typing and probiotic attributes of a new strain of *Bacillus coagulans* –Unique IS-2: a potential biotherapeutic agent. Genetic Eng Biol J. Vol. 2010, GEBJ-7.

Sudha RM, Radkar N, Maurya R. Effect of supplementation of *Bacillus coagulans* Unique IS-2 (ATCC PTA-11748) on hypercholesterolemic subjects: a clinical study. Inter J Probiotics Prebiotics. 2011b;6(2):89-94.

Sudha RM, Sunita M, Sekhar BM. Safety studies of *Bacillus coagulans* Unique IS-2 in rats: morphological, biochemical and clinical evaluations. Int J Probiotics Prebiotics. 2011a, 6(1):43-48.

Sudha RM, Yelikar KA, Deshpande S. Clinical Study of *Bacillus coagulans* Unique IS-2 (ATCC PTA-11748) in the Treatment of Patients with Bacterial Vaginosis. Indian J Microbiol. 2012a;52(3):396-9.

Tajadadi-Ebrahimi M, Bahmani F, Shakeri H, Hadaegh H, Hijijafari M, Abedi F, Asemi Z. 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. 2014;65(1):34-41.

Takagi K. Experiment on administration of "Lacris-10 for animals" to piglets. Mitsubishi-Kagaku Foods Internal Study. 1969:19-20. Unpublished.

TechnoSuruga Laboratory Co., Ltd. 16S rDNA-500 sequence analysis. Identification No. SIID17786, Mitsubishi-Kagaku Foods Corporation. 2015. Prepared November 30, 2015. Unpublished.

TechnoSuruga Laboratory Co., Ltd. Cereus Enterotoxin Detection Test Report. Identification No. SIID17786, Mitsubishi-Kagaku Foods Corporation. 2016b. Prepared March 22, 2016. Unpublished.

TechnoSuruga Laboratory Co., Ltd. DNA-DNA Hydribization Test. Identification No. SIID17786, Mitsubishi-Kagaku Foods Corporation. 2016a. Prepared June 17, 2016. Unpublished.

TechnoSuruga Laboratory Co., Ltd. 16S rDNA-500 sequence analysis. Identification No. SIID15842-03, Mitsubishi-Kagaku Foods Corporation. 2014. Prepared November 21, 2014. Unpublished.

Tortora, GJ, Funke BR, Case CL. Microbiology: An Introduction, 11th Edition. Benjamin Cummings.

United States Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition, Office of Food Additive Safety. 2012. Agency response letter, GRAS Notice No. GRN 000399. Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm314145.htm. Accessed May 8, 2015.

United States Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition, Office of Food Additive Safety. 2015. GRAS Notice No. GRN 000526. Available at: http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-foods-gen/documents/document/ucm413003.pdf. Accessed May 8, 2015.

Upadrasta A, Pitta S, Madempudi RS. Draft Genome Sequence of the Spore-Forming Probiotic Strain *Bacillus coagulans* Unique IS-2. Genome Announc. 2016 Apr 21;4(2). pii: e00225-16. doi: 10.1128/genomeA.00225-16.

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray, RGE, Stackebrandt E, Starr MP, Truper HG. Report of the ad-hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 1987;37:463-464.

Yamaguchi J, Sato Y, Koyama N. Preventive and healing effects of "Lacris for animals" on diarrhea in piglets. Mitsubishi-Kagaku Foods Internal Study. 1969:5-14. Unpublished.

Yang OO, Kelesidis T, Cordova R, Khanlou H. Immunomodulation of antiretroviral drug-suppressed chronic HIV-1 infection in an oral probiotic double-blind placebo-controlled trial. AIDS Res Hum Retroviruses. 2014;30(10):988-95.

Appendices

Appendix A. 16S rDNA Sequence Analysis of *Bacillus coagulans* SANK70258 Reports and DNA-DNA Hybridization Test Report

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16S rDNA-500 sequence analysis

Identification No.	SIID15842-03
Date this report was written	November 21, 2014
Client	[redacted]
Department	Technical Dept.
Person in charge	Assistant Manager [redacted]

METHODS

• DNA extraction	Achromopeptidase, lytic enzyme (Wako Pure Chemical Industries, Osaka, Japan)
• PCR amplification	PrimeSTAR HS DNA Polymerase (TakaraBio, Shiga, Japan)
• Cycle sequencing	BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA)
• Sequence determination	ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, AUS)
• Software	APOLLON 3.0 (TechnoSuruga Laboratory, Shizuoka, Japan)
• Homology search	APOLLON DB-BA10.0 (TechnoSuruga Laboratory, Shizuoka, Japan) International Nucleotide Sequence Database (GenBank/DDBJ/EMBL)

STRAIN INFORMATION

Isolate code	SIID	Date culture received	Source of isolate
Bacillus coagulans Lot No. 141004AA	15842-03	November. 11, 2014	—

CONCLUSION

The closest relative species based on the BLAST search (Type strain)	Similarity (%)
Bacillus coagulans NBRC 12583^T	97.8
Conclusion	**Biosafety level**
Bacillus sp.	*B. coagulans* is level 1

This report is authorized and approved by the scientist responsible on behalf of TechnoSuruga Laboratory Co., Ltd.

Scientist Responsible

[redacted]

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TechnoSuruga Laboratory Co.,Ltd.

RESULTS

Table 1. APOLLON DB-BA 10.0 BLAST Search Result: SIID15842-03

Search date: November 21, 2014

Similarity of 16S rDNA sequence between SIID15842-03 and its close relatives (30 entries)

Entry name	Strain	Accession No.	Similarity (%)	BSL
Bacillus coagulans	NBRC12583	AB271752	489/500 (97.8%)	
Bacillus shackletonii	LMG18435	AJ250318	462/502 (92.0%)	
Bacillus smithii	NBRC15311	AB271749	455/500 (91.0%)	
Bacillus ginsengihumi	Gsoil_114	AB245378	454/500 (90.8%)	
Bacillus acidiproducens	SL213	EF379274	434/468 (92.7%)	
Bacillus acidicola	105-2	AF547209	453/503 (90.1%)	
Bacillus decolorationis	LMG19507	AJ315075	449/500 (89.8%)	
Bacillus methanolicus	NCIMB13113	AB112727	455/504 (90.3%)	
Bacillus pervagus	8-4-E12	HF952773	447/496 (90.1%)	
Bacillus galliciensis	BFLP-1	FM162181	448/499 (89.8%)	
Bacillus subterraneus	DSM13966	FR733689	449/500 (89.8%)	
Bacillus boroniphilus	T-15Z	AB198719	449/500 (89.8%)	
Bacillus thermoamylovorans	LMG18084	FN666255	446/500 (89.2%)	
Bhargavaea ginsengi	ge14	EF371375	450/502 (89.6%)	
Bacillus oleronius	ATCC700005	AY988598	445/500 (89.0%)	
Bacillus thermotolerans	SgZ-8	JX261934	450/503 (89.5%)	
Bacillus jeotgali	YKJ-10	AF221061	447/500 (89.4%)	
Bacillus eiseniae	A1-2	HM035089	451/502 (89.8%)	
Bacillus thioparans	BMP-1	DQ371431	446/500 (89.2%)	
Bacillus circulans	NBRC13626	AB271747	451/502 (89.8%)	
Bacillus badius	ATCC14574	X77790	448/503 (89.1%)	
Bacillus marisflavi	TF-11	AF483624	445/500 (89.0%)	
Bacillus foraminis	CV53	AJ717382	448/501 (89.4%)	
Bacillus subtilis subsp. *subtilis*	DSM10	AJ276351	444/500 (88.8%)	
Bacillus sporothermodurans	M215	U49078	439/493 (89.0%)	
Bacillus pseudomycoides	DSM12442	AM747226	442/500 (88.4%)	
Bacillus carboniphilus	JCM9731	AB021182	445/501 (88.8%)	
Bacillus aeolius	4-1	AJ504797	442/493 (89.7%)	
Bacillus sporothermodurans	M215	U49079	437/492 (88.8%)	
Bacillus berkeleyi	KMM6244	JN187498	441/500 (88.2%)	

Remarks, BSL: Biosafety Level (above the opportunistic pathogen of level 1). The underscore (_) in the strain name indicates space.

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Table 2. GenBank/DDBJ/EMBL BLAST Search Result: SIID15842-03

Search date: November 21, 2014

Similarity of 16S rDNA sequence between SIID15842-03 and its close relatives (30 entries)

Entry name	Strain	Accession No.	Similarity (%)
Bacillus sp.	BAB-3372	KF952780	496/500 (99.2%)
Bacillus sp.	BAB-3371	KF952779	496/500 (99.2%)
Bacillus sp.	BAB-3370	KF952778	496/500 (99.2%)
Bacillus coagulans	SNZ1969	KC146407	496/500 (99.2%)
Bacillus coagulans	36D1	CP003056	496/500 (99.2%)
Bacillus coagulans	A05	HM352834	496/500 (99.2%)
Bacillus coagulans	A501	HM538423	496/500 (99.2%)
uncultured bacterium	-	HM332228	496/500 (99.2%)
uncultured bacterium	-	HM332184	496/500 (99.2%)
uncultured bacterium	-	HM332121	496/500 (99.2%)
Bacillus coagulans	NRIC 1530	AB362709	496/500 (99.2%)
Bacillus coagulans	NRIC 1528	AB362708	496/500 (99.2%)
Bacillus coagulans	NRIC 1527	AB362707	496/500 (99.2%)
Bacillus coagulans	NRIC 1526	AB362706	496/500 (99.2%)
Bacillus coagulans	IDSp	AF466695	496/500 (99.2%)
Bacillus coagulans	NBRC 3887	AB680156	496/500 (99.2%)
Bacillus coagulans	NBRC 3886	AB680155	496/500 (99.2%)
Bacillus coagulans	17C5	DQ297925	498/500 (99.6%)
Bacillus sp.	AB5216-1	GU366024	495/500 (99.0%)
uncultured bacterium	-	DQ419633	495/500 (99.0%)
Bacillus coagulans	-	AJ563373	497/500 (99.4%)
Bacillus coagulans	NBRC 3557	AB680118	495/500 (99.0%)
uncultured Firmicutes bacterium	-	FJ754735	492/496 (99.2%)
Bacillus coagulans	T5	AB240205	492/496 (99.2%)
Bacillus coagulans	NBRC 12714	AB680332	495/500 (99.0%)
Bacillus coagulans	36D1	DQ297926	494/500 (98.8%)
uncultured bacterium	-	AB367117	491/496 (99.0%)
uncultured bacterium	-	AB367092	490/496 (98.8%)
Bacillus coagulans	P4-102B	DQ297927	492/500 (98.4%)
uncultured bacterium	-	GU296471	491/500 (98.2%)

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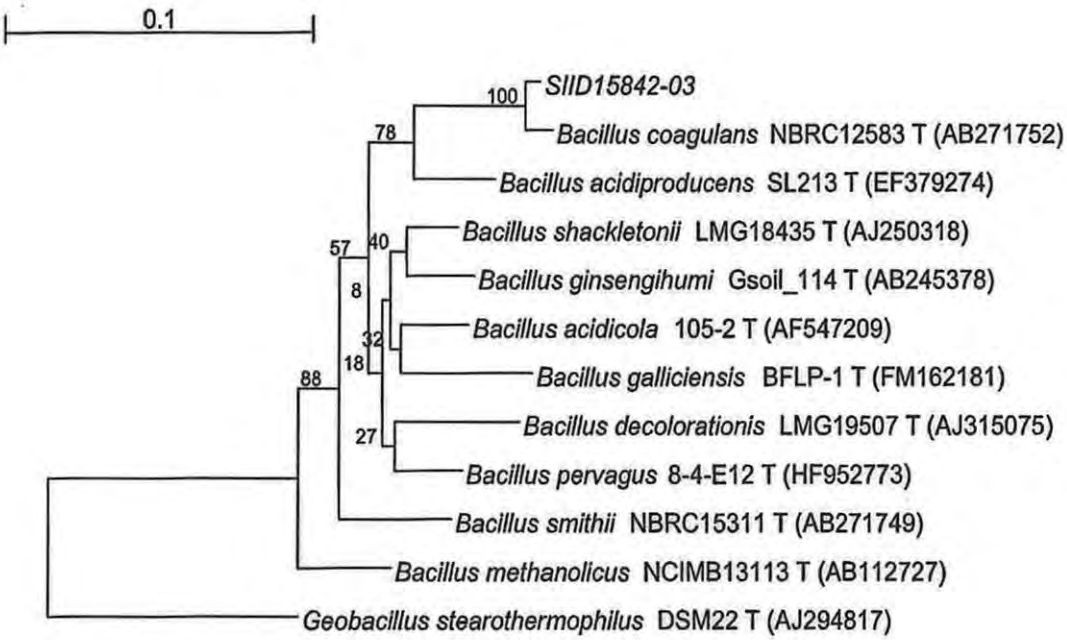

Fig. 1. Phylogenetic tree of SIID15842-03 and its related species based on the 16SrDNA partial sequences. Scale bar is shown at the upper left; bootstrap values are indicated near each cluster; T, type strain of the species; BSL, Biosafety Level (above the opportunistic pathogen of level 1).

SUPPLEMENTS

Even if the 16S rDNA sequence of isolate and type strain showed 100% similarity, it is important to determine bacterial identification, which could confirm the differences between species, based on the polyphasic taxonomic observations such as morphological, physiological, and biochemical properties; chemical analyses; and DNA–DNA hybridization. We recommend performing DNA–DNA hybridization as a final test to clarify the taxonomic relationship between isolated strains and the closest related species. In bacterial taxomony, strains showing DNA–DNA relatedness higher than 70% are defined as belonging to the same species.

The database, Apollon DB-BA, is a joint development product by TechnoSuruga Laboratory Co., Ltd. and the National Institute for Genetics. This database is composed in the 16S rDNA sequence of bacterial type strains, because it is important for bacterial identification that the features of the isolate are compared the type strain of the species. This database, Apollon DB-BA ver. 10.0 was updated at March 2014 and contains the 16S rDNA sequences of 2,127 genus, 10,548 species and 335 subspecies.

The results of the sequence identity search in the International Nucleotide Sequence Database represent the most recent data at the time when each database (GenBank/DDBJ/EMBL) was updated. Because sequence data submitted to these databases are updated on a daily basis, we recommend that the sequence homology search be repeated at a later date when you are ready to use the results of this examination.

The phylogenetic tree in this report was not premise to submission to any academic journal, since it was constructed without editing or modifying the multiple sequence alignments.

Biosafety levels (BSL) for the concerned organisms are determined at a species level. If estimation of the species is difficult, the BSL cannot be shown. In addition, the BSL does not indicate a secure level of safety because opportunistic infections have been reported in some species despite them adhering to biosafety level 1. When related species of a sample are rated above biosafety level 2 (cf. summary of this report), the sample should be handled as being above biosafety level 2. The BSLs in this report are according to the guidelines of Japanese Society for Bacteriology (http://wwwsoc.nii.ac.jp/jsb/index.html) and explanation of the each level is as below.

BSL-1: A biological agent is unlikely to cause human or animal disease by infection (this group includes agents that can be a cause of opportunistic infection).

BSL-2: A biological agent that can cause human or animal disease but cannot pose a serious risk to society. It can cause severe human disease by infection in the laboratory; however, effective prophylaxis or effective treatment is usually available.

BSL-3: A biological agent that can cause severe human disease but is unlikely to spread to the community easily.

Please contact us for technical support and further information on this report.

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TechnoSuruga Laboratory Co.,Ltd.

SIID17786

16S rDNA-Full sequence analysis

Identification No.	SIID17786
Date this report was written	30 November 2015
Client	MITSUBISHI-KAGAKU FOODS CORPORATION
Department	Technology Group Division II
Person in charge	Manager Masatoshi Takaya

METHODS

・ DNA extraction	Achromopeptidase, lytic enzyme (Wako Pure Chemical Industries, Japan)
・ PCR amplification	PrimeSTAR HS DNA Polymerase (Takara Bio, Japan)
・ Cycle sequencing	BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA)
・ Sequence determination	ChromasPro 1.7 (Technelysium, AUS)
・ Software	TechnoSuruga Lab Microbial Identification System (TechnoSuruga Laboratory, Japan)
・ Homology search	DB-BA11.0 (TechnoSuruga Laboratory) International Nucleotide Sequence Database (GenBank/DDBJ/EMBL)

STRAIN INFORMATION

Isolate code	SIID	Date culture received	Source of isolate
Bacillus coagulans SANK70258	17786	25 September 2015	Green malt

CONCLUSION

The closest relative species based on the BLAST search (Type strain)	Similarity (%)
Bacillus coagulans ATCC 7050^T 株	99.5
Conclusion	**Biosafety level**
Bacillus sp. (close to *Bacillus coagulans*)	*B. coagulans* is level 1

This report is authorized and approved by the scientist responsible on behalf of TechnoSuruga Laboratory Co., Ltd.

Scientist Responsible

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TechnoSuruga Laboratory Co.,Ltd.

SIID17786

RESULTS

Table 1. DB-BA 11.0 BLAST Search Result of SIID17786(Searched date: 7 October 2015)

Entry name	Strain	Accession No.	Similarity (%)	BSL
Bacillus coagulans	NBRC12583	AB271752	1468/1479 (99.3%)	
Bacillus shackletonii	LMG18435	AJ250318	1418/1481 (95.7%)	
Bacillus acidiproducens	SL213	EF379274	1373/1417 (96.9%)	
Bacillus ginsengihumi	Gsoil_114	AB245378	1408/1479 (95.2%)	
Bacillus acidicola	105-2	AF547209	1403/1482 (94.7%)	
Bacillus oleronius	ATCC700005	AY988598	1404/1481 (94.8%)	
Bacillus methanolicus	NCIMB13113	AB112727	1404/1484 (94.6%)	
Bacillus smithii	NBRC15311	AB271749	1396/1482 (94.2%)	
Bacillus boroniphilus	T-15Z	AB198719	1391/1479 (94.1%)	
Bacillus subterraneus	DSM13966	FR733689	1391/1479 (94.1%)	
Bacillus thermotolerans	SgZ-8	JX261934	1392/1480 (94.1%)	
Bhargavaea cecembensis	DSE10	AM286423	1392/1483 (93.9%)	
Bhargavaea ginsengi	ge14	EF371375	1391/1482 (93.9%)	
Bacillus sporothermodurans	M215	U49078	1385/1473 (94.0%)	
Bhargavaea beijingensis	ge10	EF371374	1391/1483 (93.8%)	
Bacillus thioparans	BMP-1	DQ371431	1386/1476 (93.9%)	
Bacillus sporothermodurans	M215	U49079	1383/1472 (94.0%)	
Bacillus jeotgali	YKJ-10	AF221061	1388/1479 (93.8%)	
Bacillus foraminis	CV53	AJ717382	1389/1480 (93.9%)	
Bacillus sporothermodurans	M215	U49080	1383/1473 (93.9%)	
Bacillus eiseniae	A1-2	HM035089	1389/1481 (93.8%)	
Bacillus lentus	IAM12466	D16272	1386/1481 (93.6%)	
Bacillus firmus	NBRC15306	AB271750	1383/1480 (93.4%)	
Bacillus marisflavi	TF-11	AF483624	1382/1479 (93.4%)	
Bacillus galliciensis	BFLP-1	FM162181	1380/1478 (93.4%)	
Bacillus kribbensis	BT080	DQ280367	1381/1479 (93.4%)	
Bacillus pocheonensis	Gsoil_420	AB245377	1384/1480 (93.5%)	
Bacillus niacini	IFO15566	AB021194	1386/1483 (93.5%)	
Bacillus bataviensis	LMG21833	AJ542508	1386/1481 (93.6%)	
Bacillus circulans	NBRC13626	AB271747	1386/1481 (93.6%)	

Remarks. Shaded text indicates that the sequence was used for phylogenetic analysis (Fig. 1); BSL, Biosafety Level (above the opportunistic pathogen of level 1).

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SIID17786

Table 2. GenBank/DDBJ/EMBL BLAST Search Result: SIID17786

Entry name	Strain	Accession No.	Similarity (%)
Bacillus coagulans	S-lac	CP011939	1476/1479 (99.8%)
Bacillus coagulans	HM-08	CP010525	1476/1479 (99.8%)
Bacillus sp.	BAB-3372	KF952780	1476/1479 (99.8%)
Bacillus sp.	BAB-3371	KF952779	1476/1479 (99.8%)
Bacillus sp.	BAB-3370	KF952778	1476/1479 (99.8%)
Bacillus coagulans	36D1	CP003056	1476/1479 (99.8%)
Bacillus sp.	N-16	AB618492	1476/1479 (99.8%)
Bacillus coagulans	NRIC 1527	AB362707	1476/1479 (99.8%)
Bacillus coagulans	NRIC 1526	AB362706	1476/1479 (99.8%)
Bacillus coagulans	IDSp	AF466695	1476/1479 (99.8%)
Bacillus coagulans	NBRC 3887	AB680156	1476/1479 (99.8%)
Bacillus coagulans	NRIC 1528	AB362708	1475/1479 (99.7%)
Bacillus coagulans	NBRC 3886	AB680155	1475/1479 (99.7%)
Bacillus coagulans	NRIC 1530	AB362709	1475/1479 (99.7%)
Bacillus coagulans	17C5	DQ297925	1476/1479 (99.8%)
Bacillus coagulans	-	AJ563373	1476/1479 (99.8%)
Bacillus coagulans	NBRC 3557	AB680118	1474/1479 (99.7%)
Bacillus coagulans	A05	HM352834	1474/1479 (99.7%)
Bacillus coagulans	NBRC 12714	AB680332	1474/1479 (99.7%)
Bacillus coagulans	ATCC 7050	CP009709	1472/1479 (99.5%)
Bacillus sp.	AB5216-1	GU366024	1472/1479 (99.5%)
Bacillus coagulans	36D1	DQ297926	1472/1479 (99.5%)
Bacillus coagulans	T5	AB240205	1465/1468 (99.8%)
Bacillus coagulans	P4-102B	DQ297927	1471/1479 (99.5%)
Bacillus coagulans	NBRC 106567	AB682458	1468/1479 (99.3%)
Bacillus coagulans	NBRC 12583	NR_041523	1468/1479 (99.3%)
Bacillus coagulans	C4	KJ148634	1468/1479 (99.3%)
Bacillus coagulans	2-6	NR_102791	1468/1479 (99.3%)
Bacillus coagulans	2-6	CP002472	1468/1479 (99.3%)
Bacillus coagulans	ATCC 7050	NR_115727	1467/1479 (99.2%)

Remarks. Shaded text indicates that the sequence was used for phylogenetic analysis (Fig. 1)

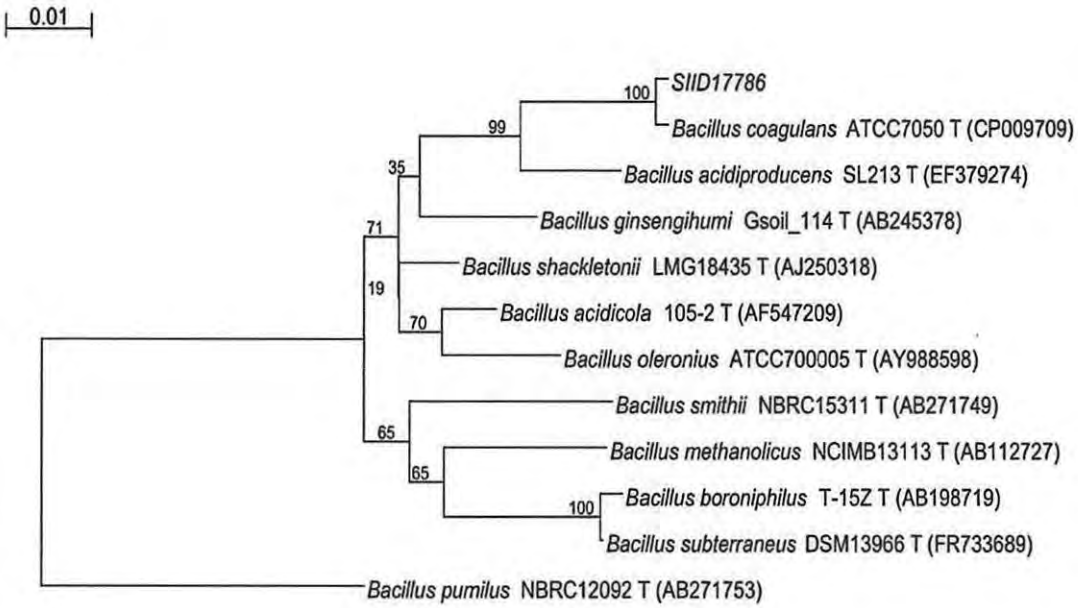

Fig. 1. Phylogenetic tree of SIID17786 and its relatives based on the 16S rDNA sequences.
Bootstrap values are indicated near each cluster; scale bar is shown at the upper left; T, type strain of the species; BSL, Biosafety Level (above the opportunistic pathogen of level 1).

SUPPLEMENTS

Even if the 16S rDNA sequence of isolate and type strain showed 100% similarity, it is important to determine bacterial identification, which could confirm the differences between species, based on the polyphasic taxonomic observations such as morphological, physiological, and biochemical properties; chemical analyses; and DNA–DNA hybridization. We recommend performing DNA–DNA hybridization as a final test to clarify the taxonomic relationship between isolated strains and the closest related species. In bacterial taxomony, strains showing DNA–DNA relatedness higher than 70% are defined as belonging to the same species.

The database, Apollon DB-BA, is a joint development product by TechnoSuruga Laboratory Co., Ltd. and the National Institute for Genetics. This database is composed in the 16S rDNA sequence of bacterial type strains, because it is important for bacterial identification that the features of the isolate are compared the type strain of the species. This database, Apollon DB-BA ver. 11.0 was updated at August 2015 and contains the 16S rDNA sequences of 2287 genus, 11,347 species and 357 subspecies.

The results of the sequence identity search in the International Nucleotide Sequence Database represent the most recent data at the time when each database (GenBank/DDBJ/EMBL) was updated. Because sequence data submitted to these databases are updated on a daily basis, we recommend that the sequence homology search be repeated at a later date when you are ready to use the results of this examination.

The phylogenetic tree in this report was not premise to submission to any academic journal, since it was constructed without editing or modifying the multiple sequence alignments.

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BSL-3: A biological agent that can cause severe human disease but is unlikely to spread to the community easily.

Please don't hesitate to ask us for technical support and further information on this report.

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TechnoSuruga Laboratory Co., Ltd.

SIID17786

DNA-DNA Hybridization Test

Identification No.	SIID17786
Date this report was written	June 17, 2016
Client	MITSUBISHI-KAGAKU FOODS CORPORATION
Department	Technology Group Division II
Person in charge	Mr. Masatoshi Takaya

CONFIDENTIAL

Confirmation

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Scientist Responsible

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TechnoSuruga Laboratory Co., Ltd.

Sample Information

Sample	SIID	Date sample received	Category of sample
Bacillus coagulans SANK70258	17786-01	—	Green malt
Bacillus coagulans NBRC12583	17786-02	November 6, 2015	NBRC strain

Notes

SIID17786-02 is the type strain of *Bacillus coagulans*.

PURPOSE

DNA-DNA hybridization with SIID17786-01 and SIID17786-02 were performed to determine the difference between the respective species.

METHODS

1. Culture condition

- Medium NBRC Medium No. 702
- Temperature 37 °C
- Time 72hr
- Other condition Aerobic condition

2. DNA extraction and purification

Genomic DNA from bacterial strains was extracted by using the modified method of Marmur [1]. Extracted crude DNAs were purified by ultracentrifugation with cesium chloride according to the method described by Hamamoto and Nakase [2].

3. Hybridization

DNA-DNA hybridization was performed using microplates according to the method of Ezaki *et al.* [3]. The fluorescence intensity in the wells was measured with a microplate reader (Genios, TECAN, Wako Pure Chemical Industries, Ltd., Osaka).

RESULTS

The DNA-DNA hybridization was performed in order to determine the difference between the species SIID17786-01 and SIID17786-02. The results and the average of three hybridization assays are shown in Table 1. The DNA-DNA similarity values by three assays were between 55% and 64 %.

Table 1. The results of DNA-DNA hybridization.

1st	Probe (labeled DNA) (%)	
Fixed DNA	SIID17786-01	SIID17786-02
SIID17786-01	100	56
SIID17786-02	62	100

2 nd	Probe (labeled DNA) (%)	
Fixed DNA	SIID17786-01	SIID17786-02
SIID17786-01	100	57
SIID17786-02	64	100

3 rd	Probe (labeled DNA) (%)	
Fixed DNA	SIID17786-01	SIID17786-02
SIID17786-01	100	55
SIID17786-02	60	100

Average	Probe (labeled DNA) (%)	
Fixed DNA	SIID17786-01	SIID17786-02
SIID17786-01	100	55
SIID17786-02	62	100

REFERENCE

1) **Marmur J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3,** 208–218.

2) **Hamamoto M. & Nakase T. (1995).** Ballistosporous yeasts found on the surface of plant materials collected in New Zealand. *Antonie van Leewenhoek* **67,**151–171.

3) **Ezaki T., Hashimoto Y., & Yabuuchi E. (1989).** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39,** 224–229.

4) **Wayne L. G., Brenner D. J., Colwell R. R., Grimont P. A. D., Kandler O., Krichevsky M. I., Moore L. H., Moore W. E. C., Murray R. G. E. & other authors (1987).** Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37,** 463–464.

5) **Johnson J. L. (1984).** Nucleic acids in bacterial classification. In *Bergey's Manual of Systematic Bacteriology*. **Vol. 1,** pp. 8–11. New York, Springer.

SUPPLEMENT

More than 60% of DNA-DNA similarity values among bacterial species indicates that the species could be defined as a same species including subspecies[5]. However, the interpretation from the result of DNA-DNA homology is considered comprehensively with all other characters such as phylogenetic taxonomy based on 16S rDNA nucleotide sequence analysis, physiological/biochemical properties and chemotaxonomic properties of bacterial components.

Please contact us for technical support and further information on this report.

E-mail: tsl-contact@tecsrg.co.jp, Free fax: +(81)-120-543-580.

Appendix B. Compositional Analyses of *Bacillus coagulans* and the LACRIS-S Preparation

MITSUBISHI-KAGAKU FOODS CORPORATION
1-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100-8251, Japan

August 4, 2015

ANALYTICAL REPORT

Product	**: Bacillus coagulans**
Lot No.	**: 141004AA**
Manufacturing date	**: October 4, 2014**

TEST ITEMS	**RESULTS**
Description	**; Light brown powder having a slightly unique odor**
Loss on drying	**; 7.8 %**
Bacillus coagulans spore count (BCP)	**; $5{,}090\times10^{8}$ cfu/g**
Total bacteria (SCDA)	**; Less than 10 cfu/g**
Coliforms	**; Negative/g**
<REFFERENCE TEST>	
Identification	**; Conforms**
Fungus	**; Less than 10 cfu/g**
E. coli	**; Negative/2.22g**
Salmonella	**; Negative/25g**
Listeria monocytogenes	**; Negative/25g**
Enterobacteriacea	**; Less than 10 cfu/g**
Arsenic(as As)	**; Not detected (<0.1ppm)**
Lead	**; Not detected (<0.05ppm)**
Cadmium	**; 0.02ppm**
Mercury	**; Not detected (<0.01ppm)**

[redacted]

Tetsuya Fukazawa
Group Manager
Quality Assurance Department

MITSUBISHI-KAGAKU FOODS CORPORATION
1-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100-8251, Japan

December 10, 2015

ANALYTICAL REPORT

Product	**: Bacillus coagulans**
Lot No.	**: 150221AA**
Manufacturing date	**: February 21, 2015**

TEST ITEMS		**RESULTS**
Description	;	**Light brown powder having a slightly unique odor**
Loss on drying	;	**5.7 %**
Bacillus coagulans spore count (BCP)	;	**$6{,}050\times10^{8}$ cfu/g**
Total bacteria (SCDA)	;	**Less than 10 cfu/g**
Coliforms	;	**Negative/g**
<REFFERENCE TEST>		
Identification	;	**Conforms**
Fungus	;	**Less than 10 cfu/g**
E. coli	;	**Negative/2.22g**
Salmonella	;	**Negative/25g**
Listeria monocytogenes	;	**Negative/25g**
Enterobacteriacea	;	**Less than 10 cfu/g**
Arsenic(as As)	;	**0.1ppm**
Lead	;	**Not detected (<0.05ppm)**
Cadmium	;	**0.01ppm**
Mercury	;	**Not detected (<0.01ppm)**

[redacted]

Tetsuya Fukazawa
Group Manager
Quality Assurance Department

MITSUBISHI-KAGAKU FOODS CORPORATION
1-1, Marunouchi 1-chome, Chiyoda-ku, Tokyo 100-8251, Japan

December 10, 2015

ANALYTICAL REPORT

Product	**: Bacillus coagulans**
Lot No.	**: 150307AA**
Manufacturing date	**: March 7, 2015**

TEST ITEMS	**RESULTS**
Description	**; Light brown powder having a slightly unique odor**
Loss on drying	**; 6.3 %**
Bacillus coagulans spore count (BCP)	**; $4{,}880\times10^{8}$ cfu/g**
Total bacteria (SCDA)	**; Less than 10 cfu/g**
Coliforms	**; Negative/g**
<REFFERENCE TEST>	
Identification	**; Conforms**
Fungus	**; Less than 10 cfu/g**
E. coli	**; Negative/2.22g**
Salmonella	**; Negative/25g**
Listeria monocytogenes	**; Negative/25g**
Enterobacteriacea	**; Less than 10 cfu/g**
Arsenic(as As)	**; 0.1ppm**
Lead	**; Not detected (<0.05ppm)**
Cadmium	**; 0.02ppm**
Mercury	**; Not detected (<0.01ppm)**

Tetsuya Fukazawa
Group Manager
Quality Assurance Department

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December 10, 2015

CERTIFICATE OF ANALYSIS

Specification	**: MITSUBISHI-KAGAKU FOODS Standard**
Product	**: Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No.	**: A11015**
Manufacturing date	**: November 25, 2014**
Expiration date	**: November 24, 2016**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor .	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	1.1%
Lactic acid producing ability	;	Not less than 10 mL	:	20 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^{9} cfu/g	:	7.3×10^{9} cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
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December 10, 2015

CERTIFICATE OF ANALYSIS

Specification : **MITSUBISHI-KAGAKU FOODS Standard**
Product : **Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No. : **B10238**
Manufacturing date : **March 24, 2015**
Expiration date : **March 23, 2017**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor.	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	2.1%
Lactic acid producing ability	;	Not less than 10 mL	:	19 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^9 cfu/g	:	7.1×10^9 cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
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December 18, 2015

CERTIFICATE OF ANALYSIS

Specification	**: MITSUBISHI-KAGAKU FOODS Standard**
Product	**: Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No.	**: B10240**
Manufacturing date	**: March 25, 2015**
Expiration date	**: March 24, 2017**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor .	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	2.5%
Lactic acid producing ability	;	Not less than 10 mL	:	19 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^9 cfu/g	:	6.4×10^9 cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
Group Manager
Quality Assurance Department

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April 4, 2016

CERTIFICATE OF ANALYSIS

Specification : **MITSUBISHI-KAGAKU FOODS Standard**
Product : **Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No. : **B11299**
Manufacturing date : **December 17, 2015**
Expiration date : **December 16, 2017**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor.	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	1.7%
Lactic acid producing ability	;	Not less than 10 mL	:	19 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^9 cfu/g	:	6.1×10^9 cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
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April 4, 2016

CERTIFICATE OF ANALYSIS

Specification : **MITSUBISHI-KAGAKU FOODS Standard**
Product : **Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No. : **B11201**
Manufacturing date : **December 17, 2015**
Expiration date : **December 16, 2017**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor.	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	1.7%
Lactic acid producing ability	;	Not less than 10 mL	:	19 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^{9} cfu/g	:	6.2×10^{9} cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
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Quality Assurance Department

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April 4, 2016

CERTIFICATE OF ANALYSIS

Specification	**: MITSUBISHI-KAGAKU FOODS Standard**
Product	**: Spore Forming Lactic acid bacteria (LACRIS-S)**
Lot No.	**: C10103**
Manufacturing date	**: January 26, 2016**
Expiration date	**: January 25, 2018**

TEST ITEMS		SPECIFICATIONS		RESULTS
Description	;	White to light yellow powder having a unique odor .	:	Conforms
Identification	;	Conforms	:	Conforms
Loss on drying	;	Not more than 8.0 %	:	3.4%
Lactic acid producing ability	;	Not less than 10 mL	:	19 mL
Bacillus coagulans spore count	;	Not less than 5.0×10^9 cfu/g	:	10.0×10^9 cfu/g
Arsenic	;	Not more than 0.5ppm	:	Not detected (<0.1ppm)
Cadmium	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Lead	;	Not more than 0.5ppm	:	Not detected (<0.05ppm)
Mercury	;	Not more than 0.5ppm	:	Not detected (<0.01ppm)
Total bacteria	;	Not more than 1000 cfu/g	:	Conforms
Fungus	;	Not more than 500 cfu/g	:	Conforms
Coliforms	;	Negative/g	:	Conforms

[redacted]

Tetsuya Fukazawa
Group Manager
Quality Assurance Department

Appendix C. Antibiotic Susceptibility Testing of *B. coagulans* SANK 70258 in a Spores Preparation

Antibiotic Susceptibility Test of *Bacillus coagulans* SANK70258 Spore Preparation (Product Name: LACRIS-S)

FINAL REPORT

Prepared: July 19, 2016

Hashima Laboratory, Nihon Bioresearch Inc.

1. Contents

2. Signature of Person Preparing Final Report

Study No.: 360021

Title: Antibiotic Susceptibility Test of *Bacillus coagulans* SANK70258 Spore Preparation (Product Name: LACRIS-S)

Hashima Laboratory, Nihon Bioresearch Inc.

Study Director _____(signed)_____ (seal) July 19, 2016
Ryusuke Sakuma

3. Title of the Study

Antibiotic Susceptibility Test of *Bacillus coagulans* SANK70258 Spore Preparation (Product Name: LACRIS-S)

4. Study Number

360021

5. Sponsor

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6. Test Facility

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TEL: +81-584-51-2737; FAX: +81-584-51-0856

7. Objective of the Study

To assess the antibiotic susceptibility of *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) by disk diffusion method and microdilution method.

8. Guidelines Followed

No guidelines followed.

9. Study Director

Ryusuke Sakuma

10. Study Schedule

Start of the study	April 13, 2016
Start of the test	April 18, 2016
Completion of the test	April 23, 2016
Completion of the study	July 19, 2016

11. Archives

All data (original of the protocol, raw data, and original of the final report) generated in the present study at the test facility will be stored in the archives of Hashima Laboratory, Nihon Bioresearch Inc. for 10 years after preparation of the final report. Their subsequent disposition will be determined by mutual agreement with the sponsor.

12. Unforeseeable Circumstances That Might Have Affected the Reliability of the Study and Deviations from the Protocol

There were no unforeseeable circumstances that might have adversely affected the reliability of the study during the study period, and there were no deviations from the protocol.

13. Study Personnel and Work Responsibility

Study Director: Ryusuke Sakuma

Preparation of the protocol, test implementation, and preparation of the final report

Study Personnel: Tatsumi Inoue

Test implementation

14. Summary

The antibiotic susceptibility of *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was assessed by the disk diffusion method and microdilution method.

In the disk diffusion method, 18 antibiotics (streptomycin, gentamicin, bacitracin, novobiocin, polymyxin, cefaclor, ciprofloxacin, rifampicin, chloramphenicol, tetracycline, erythromycin, kanamycin, colistin, nalidixic acid, clindamycin, cefoxitin, doxycycline, and penicillin) were tested.

In the microdilution method, 18 antibiotics (oxacillin, ampicillin, cefazolin, cefmetazole, flomoxef, imipenem, gentamicin, arbekacin, minocycline, cefoxitin, erythromycin, clindamycin, vancomycin, teicoplanin, linezolid, fosfomycin, sulfamethoxazole-trimethoprim, and levofloxacin) were tested.

In the test by the disk diffusion method, *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was judged to be sensitive to all antibiotics from the mean diameter of the zone of growth inhibition around each of the antibiotic disks measured. The minimum inhibitory concentrations of flomoxef and linezolid were low at 1 and 0.5 μg/mL, respectively, in the test by the microdilution method. No bacterial growth was noted even at the lowest concentration for the other antibiotics; the minimum inhibitory concentrations were below the lowest concentrations tested.

As described above, *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was susceptible to all antibiotics in the disk diffusion method and microdilution method. Therefore, it was suggested that *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) has little possibility of having a transferable antibiotic-resistant gene.

15. Introduction

The antibiotic susceptibility of *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was assessed by the disk diffusion method and microdilution method.

16. Materials and Methods

16.1. Test Bacteria

16.1.1. Test Bacteria

Name:	*Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S)
Lot No.:	B10760
Storage Conditions:	Stored under refrigerated conditions.
Temperature:	2.0 to 8.0°C
Storage Site:	Stored in a refrigerator/freezer (MPR-215FS, SANYO Electric Co., Ltd.) in the test article storage room of Kisosansen Laboratory.
Supplier:	Mitsubishi-Kagaku Foods Corporation

16.1.2. Incubation

The test bacteria (1 g) were measured out (electronic balance: XP205DRV, Mettler-Toledo GmbH) and put into a 50-mL plastic tube. Then 25 mL of physiological saline was added, and the mixture was stirred well with a mixer (SI-2086, Scientific Industries) for 5 minutes. Another 10 mL of saline was added to the mixture, and the mixture was stirred well with a mixer for 5 minutes. Saline was further added to the mixture to make 100 mL of a suspension. The suspension was shaken well, put in water at 75°C for 30 minutes, and cooled in ice immediately to prepare a sample suspension. A plate count agar with BCP (Plate Count Agar with BCP "Nissui," Nissui Pharmaceutical Co., Ltd.) was streaked with the sample suspension and incubated for 3 days in an incubator (MTR-251, SANYO Electric Co., Ltd.) set at 37°C.

16.2. Antibiotic Susceptibility Test

16.2.1. Disk Diffusion Method

BD Sensi-Disc (Becton, Dickinson and Company), a kit for the disk diffusion method, was used.

16.2.1.1. Antibiotics Used for Test

(1) Streptomycin (content: 300 μg)
(2) Gentamicin (content: 120 μg)
(3) Bacitracin (content: 10 U)
(4) Novobiocin (content: 30 μg)
(5) Polymyxin (content: 300 U)
(6) Cefaclor (content: 30 μg)
(7) Ciprofloxacin (content: 5 μg)
(8) Rifampicin (content: 5 μg)
(9) Chloramphenicol (content: 30 μg)
(10) Tetracycline (content: 30 μg)
(11) Erythromycin (content: 15 μg)
(12) Kanamycin (content: 30 μg)
(13) Colistin (content: 10 μg)
(14) Nalidixic acid (content: 30 μg)
(15) Clindamycin (content: 2 μg)
(16) Cefoxitin (content: 30 μg)
(17) Doxycycline (content: 30 μg)
(18) Penicillin (content: 10 U)

16.2.1.2. Preparation of Bacterial Suspension

In the generated colonies, 5 colonies which turned yellow were collected with a platinum loop, and the colonies were suspended in 5 mL of sterile physiological saline. Turbidity of the suspension was adjusted macroscopically to 0.5 following McFarland Standards.

16.2.1.3. Disk Diffusion Method

A sterile cotton swab was soaked in the prepared bacterial suspension, and the plate count agar with BCP was streaked evenly with the swab. The plate count agar with BCP was rotated 60 degrees and streaked again; these procedures were repeated twice. The surface of the agar was dried for about 5 minutes. Then Sensi-Disc was put on the agar, and the agar was incubated for about 24 hours in an incubator set at 37°C. For the test, 3 Sensi-Discs were used for each antibiotic.

16.2.1.4. Judging Method

The diameter of zone of growth inhibition around each of the antibiotic disks was measured with a caliper after incubation, and the mean value for each disk was calculated. Susceptibility was judged following the criteria (performance standards for antimicrobial disk susceptibility, CLSI [Clinical and Laboratory Standards Institute]) shown below. After judgment, the agar was autoclaved (at 121°C for 15 minutes, LSX-500, Tomy Seiko Co., Ltd.) and discarded.

Antibiotic	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Streptomycin	6	7 - 9	10
Gentamicin	6	7 - 9	10
Bacitracin	8	9 - 12	13
Novobiocin	17	18 - 21	22
Polymyxin	11	–	12
Cefaclor	14	15 - 17	18
Ciprofloxacin	15	16 - 20	21
Rifampicin	16	17 - 19	20
Chloramphenicol	12	13 - 17	18
Tetracycline	11	12 - 14	15
Erythromycin	13	14 - 22	23
Kanamycin	13	14 - 17	18
Colistin	10	–	11
Nalidixic acid	13	14 - 18	19
Clindamycin	15	16 - 18	19
Cefoxitin	14	15 - 17	18
Doxycycline	10	11 - 13	14
Penicillin	14	–	15

16.2.2. Microdilution Method

Dry Plate 'Eiken' (Eiken Chemical Co., Ltd.), a kit for the microdilution method, was used. Dry Plate 'Eiken' is a reagent prepared in compliance with performance standards set by CLSI.

16.2.2.1. Antibiotic Sequences on Plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	MPIPC 4	2	1	0.5	0.25	0.12	MINO 8	4	2	CFX 16	8	4
B	ABPC 16	8	4	0.5	0.25	0.12	EM 4	2	1	0.5	0.25	0.12
C	CEZ 16	8	4	2	1	0.5	CLDM 2	1	0.5	0.25	0.12	0.06
D	CMZ 32	16	8	4	2	1	VCM 16	8	4	2	1	0.5
E	FMOX 16	8	4	2	1	0.5	TEIC 16	8	4	2	1	0.5
F	IPM 8	4	2	1	0.5	0.25	LZD 8	4	2	1	0.5	0.25
G	GM 8	4	2	1	0.5	0.25	FOM 128	64	32	ST 38/2	19/1	9.5/0.5
H	ABK 8	4	2	1	0.5	0.25	LVFX 4	2	1	0.5	0.25	Control

(μg/mL)

MPIPC: Oxacillin, ABPC: ampicillin, CEZ: cefazolin, CMZ: cefmetazole, FMOX: flomoxef, IPM: imipenem, GM: gentamicin, ABK: arbekacin, MINO: minocycline, CFX: cefoxitin, EM: erythromycin, CLDM: clindamycin, VCM: vancomycin, TEIC: teicoplanin, LZD: linezolid, FOM: fosfomycin, ST: sulfamethoxazole-trimethoprim, and LVFX: levofloxacin.

16.2.2.2. Preparation of Bacterial Suspension

In the generated colonies, 5 colonies which turned yellow were collected with a platinum loop, and the colonies were suspended in 5 mL of sterile physiological saline. Turbidity of the suspension was adjusted macroscopically to 1 following McFarland Standards. Mueller-Hinton broth was added to 0.025 mL of the prepared bacterial suspension, and the bacteria were suspended uniformly to make a bacterial suspension for inoculation.

16.2.2.3. Microdilution Method

The bacterial suspension for inoculation, 0.05 mL, was inoculated to each well of the plate, and the suspension in the plate was incubated for 2 days in an incubator set at 37°C. For the test, 2 plates were used.

16.2.2.4. Judging Method

Bacterial growth was confirmed after incubation, and the minimum inhibitory concentrations (MIC) were calculated. The plates used for incubation were autoclaved (at 121°C for 15 minutes) and discarded.

Criteria

Positive: Opacity or deposit of 1 mm or more in diameter was noted macroscopically.

Deposit was less than 1 mm in diameter, but 2 or more massive deposits were noted.

Negative: No opacity or deposit was noted macroscopically.

Deposit was noted, but there was only 1 deposit of less than 1 mm in diameter.

17. Results

17.1. Disk Diffusion Method

Results of the test are shown in Table 1.

Bacillus coagulans SANK70258 spore preparation (product name: LACRIS-S) was sensitive to all antibiotics.

17.2. Microdilution Method

Results of the test are shown in Table 2.

The minimum inhibitory concentrations of flomoxef and linezolid were 1 and 0.5 μg/mL, respectively. No bacterial growth was noted for the other antibiotics; the minimum inhibitory concentrations were below the lowest concentrations.

18. Discussion

The antibiotic susceptibility of *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was assessed by the disk diffusion method and microdilution method.

In the test by the disk diffusion method, *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was judged to be sensitive to all antibiotics from the mean diameter of zone of growth inhibition around each of the antibiotic disks measured. The minimum inhibitory concentrations were low for all antibiotics in the test by the microdilution method.

As described above, *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) was susceptible to all antibiotics in the disk diffusion method and microdilution method. Therefore, it was suggested that *Bacillus coagulans*

SANK70258 spore preparation (product name: LACRIS-S) has little possibility of having a transferable antibiotic-resistant gene.

Table 1. Antibiotic susceptibility for *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) in disk diffusion method

Antibiotics	Susceptibility
Streptomycin	S
Gentamicin	S
Bacitracin	S
Novobiocin	S
Polymyxin	S
Cefaclor	S
Ciprofloxacin	S
Rifampicin	S
Chloramphenicol	S
Tetracycline	S
Erythromycin	S
Kanamycin	S
Colistin	S
Nalidixic acid	S
Clindamycin	S
Cefoxitin	S
Doxycycline	S
Penicillin	S

S: Sensitive, I: intermediate, R: resistant.

Table 2. Antibiotic susceptibility for *Bacillus coagulans* SANK70258 spore preparation (product name: LACRIS-S) in microdilution method

Antibiotics	MIC (μg/mL)
Oxacillin	<0.12
Ampicillin	<0.12
Cefazolin	<0.5
Cefmetazole	<1
Flomoxef	1.0
Imipenem	<0.25
Gentamicin	<0.25
Arbekacin	<0.25
Minocycline	<2
Cefoxitin	<4
Erythromycin	<0.12
Clindamycin	<0.06
Vancomycin	<0.5
Teicoplanin	<0.5
Linezolid	0.5
Fosfomycin	<32
Sulfamethoxazole-trimethoprim	<9.5/0.5
Levofloxacin	<0.25

Appendix D. Duopath® Cereus Enterotoxin Detection Test Report

Cereus Enterotoxin Detection Test Report

Identification No.	SIID17786
Date this report was written	22 March 2016
Client	MITSUBISHI-KAGAKU FOODS CORPORATION Technology Group Division II
Person in charge	Manager Masatoshi Takaya

CONFIDENTIAL

Confirmation

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Scientist Responsible

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Isolate Information

Isolate code	SIID	Date culture received	Source of isolate
Bacillus coagulans SANK70258	17786-01	3 February 2016	green malt

Notes

PURPOSE

Bacillus cereus enterotoxin production of the sample (SIID17786-01) is detected.

METHOD

1. Culture condition

The bacterial strain was cultured under the following conditions.

- Medium CGY broth (Merk, Germany) (with 1.0 % glucose)
- Temperature 37℃
- Time 4 hr

2. Kit

Duopath Cereus Enterotoxins (Merk, Germany)

RESULT

Result of the test is shown in the figure 1.

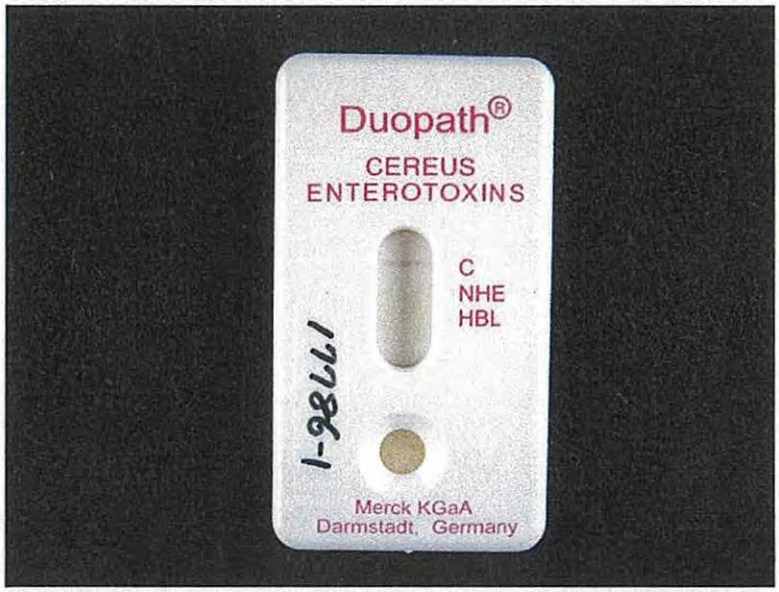

Fig. 1. Result of cereus enterotoxin detection test

C: control

NHE: non-hemolytic enterotoxin

HBL: hemolysin BL enterotoxin

DISCUSSION

By the result of cereus enterotoxin detection test, SIID17786-01 was considered negative for NHE and HBL.

CONCLUSION

We concluded the SIID17786-01 is a *Bacillus cereus* enterotoxin non-producing strain.

Exhibit: Report of the Expert Panel

EXPERT PANEL OPINION

The Generally Recognized As Safe (GRAS) Status of the Use of *Bacillus coagulans* SANK 70258 Spores Preparation (LACRIS-S) in Select Foods

Introduction

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), was specially convened by Mitsubishi-Kagaku Foods Corporation ("MKF"), to evaluate the safety and "generally recognized as safe" ("GRAS") status of the intended use of the *Bacillus coagulans* (*"B. coagulans"*) SANK 70258 spores preparation referred to as LACRIS-S in select foods. For purposes of this review, "safe" or "safety" means that there is "a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use," as defined by the US Food and Drug Administration (FDA) in 21 CFR 570.3(i).

Exponent, Inc. ("Exponent") performed a comprehensive search of the scientific literature, through June 8, 2016, relating to the safety of the *B. coagulans* SANK 70258 spores preparation. Exponent summarized the results of the literature search and prepared a safety dossier, "Documentation Supporting the Generally Recognized as Safe (GRAS) Determination for the Use of *Bacillus coagulans* SANK 70258 Spores Preparation (LACRIS-S) in Select Foods" for consideration by the Expert Panel.

The Expert Panel (Drs. Pariza, Tarka, and Thomas) critically evaluated Exponent's safety documentation (the dossier), and other available data and information that the members of the Expert Panel believed to be pertinent to the safety of the *B. coagulans* SANK 70258 spores preparation intended for use in select foods.

On July 5, 2016, the Expert Panel convened via teleconference, and independently, jointly, and unanimously concluded that the *B. coagulans* SANK 70258 spores preparation, produced consistent with current good manufacturing practice ("cGMP") and meeting the stated specifications, is safe for its intended use in foods. The Expert Panel further concluded unanimously that the intended use of the *B. coagulans* SANK 70258 spores preparation is GRAS based on scientific procedures. It is also the unanimous consensus opinion of this Expert Panel that other qualified experts would concur with these conclusions.

Summarized below are the data, information, and interpretive analysis supporting the Expert Panel's conclusions.

Description

LACRIS-S is the trade name for a proprietary preparation of *B. coagulans* strain SANK 70258, a spore-forming lactic acid bacteria. The LACRIS-S preparation consists of spores of *B.*

coagulans diluted in lactose. The LACRIS-S preparation is made using a production process with controls for manufacturing of the microorganism to ensure suitability for use in food and the product meets specifications appropriate for a food ingredient. All substances used in the manufacture of LACRIS-S are commonly used in the manufacture of fermented foods and are used in accord with cGMP. The LACRIS-S preparation contains a minimum *B. coagulans* spores count of 5.0×10^9 CFU per gram.

Intended Use

B. coagulans SANK 70258 in the LACRIS-S spores preparation is proposed for use (up to 2×10^9 CFU *B. coagulans*/serving) as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups.

The proposed use of LACRIS-S, resulting in a concentration of *B. coagulans* SANK 70258 of up to 2×10^9 CFU/serving as an ingredient in select food categories, is identical to the proposed use of spores preparations detailed in four GRAS Notification ("GRN"), namely preparations containing *B. coagulans* strain GBI-30, 6086 (GRN 399), *B. coagulans* strain Unique IS2 (GRN 526), *B. coagulans* SNZ1969 (GRN 597), and *B. coagulans* SBC37-01 (GRN 601). In the GRN for *B. coagulans* GBI-30, 6086 (GRN 399), the notifier calculated the maximum estimated daily intake ("EDI") of the organism from the intended use of up to 2×10^9 CFU/serving in the United States at 36.4×10^9 CFU per day. This estimate was based on data from the USDA Center for Nutrition Policy and Promotion showing that the maximum number of food servings consumed per day is 18.2 (among the population of males ages 51 years and older) and the assumption that the maximum intended use of the organism would be added to each serving of food consumed. The FDA had no questions regarding the proposed use levels and the resulting EDI as calculated originally by the notifier or in use of a substitutional approach as proposed in the subsequent GRN for identical uses of a *B. coagulans* spores preparation (GRNs 526, 597, 601).

Assuming that the proposed use of *B. coagulans* SANK 70258 is substitutional for use of preparations of *B. coagulans* spores from strains GBI-30, 6086, Unique IS2, SNZ1969, or SBC37-01, the maximum EDI of all strains of *B. coagulans* combined is 36.4×10^9 CFU per day.

Safety

The safety of the proposed use of the *B. coagulans* SANK 70258 spores preparation was evaluated based on both evidence and data provided in four safety reviews previously developed to establish the GRAS status of the use of other *B. coagulans* strains (GRNs 399, 526, 597, 601), 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 provided by MKF on the phenotypic and genotypic analysis of the organism as well as a series of unpublished supportive

safety evaluations. The totality of evidence, in combination with information on the established history of use of the species, was evaluated to determine the safety of the strain in a spores preparation.

Bacillus species are potential spore-forming microorganisms that are naturally occurring in soil, and *B. coagulans* species are used in the fermentation of ethnic foods such as ugba in Nigeria. Use of *B. coagulans* for health dates back approximately 50 years or more with the introduction of the *B. coagulans* SANK 70258 strain in a spores preparation for probiotic foods in Japan. More recently, spores preparations of *B. coagulans* have been the subject of four (4) GRAS notifications (GRNs 399, 526, 597, 601). *B. coagulans* SNZ1969, the subject of GRN 597, is closely related to *B. coagulans* SANK 70258. The FDA had no questions concerning the conclusion that each spores preparation of *B. coagulans* was GRAS under the intended conditions of use. In addition to the recognition of GRAS uses in foods, *B. coagulans* may be used for the production of insoluble enzyme preparations as established by Codex, the U.S. FDA, and Health Canada. *B. coagulans* has been granted Qualified Presumption of Safety status which suggests that use of *B. coagulans* in foods is considered safe, and Food Standards Australia New Zealand identified no safety concerns with the species. *B. coagulans* is classified by ATCC as a Biosafety Level One ("BSL-1") organism based on U.S. Public Health Service Guidelines, indicating the organism is not known to cause disease in healthy human adults.

Data from conventional microscopic observation and biochemical characterization in combination with genotypic identification, including a 16S rDNA full sequence analysis and comparison to known gene sequences, provides evidence that the strain used in the production of the LACRIS-S spores preparation and referred to as strain SANK 70258 is of the *B. coagulans* species. The *B. coagulans* strain in LACRIS-S was identified as a *Bacillus* species with similarity to known *B. coagulans* strains of 99.5% (with ATCC 7050) and 99.8% (with other multiple strains) in a GenBank/DDBJ/EMBL BLAST database search of the full 16S rDNA sequence. The *B. coagulans* SANK 70258 strain is routinely assessed with morphological and physiological tests to ensure consistency, it is examined annually for genetic stability, and microscopic checks throughout the production process are conducted to confirm the absence of morphological abnormity.

B. coagulans is susceptible to a variety of common antibiotics and genetic testing on *B. coagulans* SNZ1969, a strain of *B. coagulans* closely related to *B. coagulans* SANK 70258, indicates that the strain showed no evidence for the presence of plasmids and consequently is not likely to gain and transfer plasmid-borne antibiotic-resistance genes. No information was identified indicating that oral ingestion of *B. coagulans* causes infection, and testing of the *B. coagulans* SANK 70258 strains demonstrates that the strain is negative for non-hemolytic enterotoxin and hemolysin BL enterotoxin.

B. coagulans SANK 70258 and similar strains have been tested in a range of pre-clinical and clinical studies. The *B. coagulans* strains tested in pre-clinical and clinical studies are very similar to *B. coagulans* SANK 70258 and these data can be used to support the safety of *B. coagulans* SANK 70258 in a spores preparation. Studies with other strains of *B. coagulans* indicate that the organism is not acutely toxic or irritating, is not genotoxic, and is not a reproductive toxin. In a one-year chronic toxicity study of *B. coagulans* GBI-30, 6086 the no-observed adverse effect level (NOAEL) was determined by the investigators to be 1.34×10^{11}

CFU/kg bw/d, which corresponds to an acceptable daily intake ("ADI") of 1.34 x 10^9 CFU/kg bw/d after application of an uncertainty factor of 100. Collectively, the findings from these studies provide pivotal evidence to support the safety of consumption of *B. coagulans* at an intake up to 1.34 x 10^9 CFU/kg bw/d. This ADI is corroborated by the ADI derived from the unpublished 90-day study of the same strain of *B. coagulans* contained in LACRIS-S preparation, strain SANK 70258, which was 1.02 x 10^9 CFU/kg bw/d based on the highest dose tested.

There were no reports of serious adverse effects or observed safety concerns in published clinical trials with *B. coagulans* SANK 70258, and in more than 20 published clinical trials with other strains of *B. coagulans* and species of unspecified strain from daily intake of *B. coagulans*. Daily intake of *B. coagulans* was up to approximately 20 x 10^9 CFU. The longest study of *B. coagulans* supplementation, a study of infants receiving 0.1 x 10^9 CFU/d, spanned a period of 1 year. In several studies in adults, the period of intervention was in the range of 8 to approximately 13 weeks. Results from these studies in both healthy and compromised individuals indicate that use of *B. coagulans* presented no evidence of pathogenicity or toxicity and was suitably tolerated.

Summary and Conclusion

The safety of the proposed use of *B. coagulans* SANK 70258 in a spores preparation (up to 2 x 10^9 CFU/serving) includes use as an ingredient in: baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings and syrups. The estimated daily intake of *B. coagulans* SANK 70258 from these uses is 36.4 x 10^9 CFU per day. *B. coagulans* is classified as BSL-1 organism, indicating the organism is not known to cause disease in healthy human adults. The available evidence using conventional phenotypic analysis in combination with genotypic analysis confirms the identity of the *B. coagulans* SANK 70258 microorganism as a strain of *B. coagulans*. Studies of several strains of *B. coagulans* indicate that the organism is not acutely toxic or irritating, is not genotoxic, and is not a reproductive toxin. Pre-clinical studies of *B. coagulans* SANK 70258 corroborate these findings. Clinical studies in healthy and compromised individuals indicate that use of *B. coagulans* – including SANK 70258 – presented no evidence of pathogenicity or toxicity and was generally well tolerated. The available evidence also shows that *B. coagulans* is not resistant to many common antibiotics, and the strain is not known to have virulence activity. The analytical data on *B. coagulans* are supported by decades of use with no reported adverse effects. It is therefore reasonable to conclude that the proposed use of *B. coagulans* SANK 70258 in a spores preparation is safe within the meaning of the FD&C Act, i.e., meets the standard of reasonable certainty of no harm.

Expert Panel Conclusion

We, the undersigned expert panel members, have independently and collectively critically evaluated published and unpublished data and information summarized above and unanimously conclude that the intended use of the *Bacillus coagulans* SANK 70258 spores preparation (LACRIS-S) in a concentration of *B. coagulans* SANK 70258 of up to 2 x 10^9 CFU/serving as an ingredient in select food categories, produced consistent with cGMP and meeting appropriate food-grade specifications as presented in the supporting dossier ("Documentation Supporting the GRAS Determination for the Use of *Bacillus coagulans* SANK 70258 Spores Preparation (LACRIS-S) in Select Foods") is safe.

We, the undersigned expert panel members, further unanimously conclude that the intended use of *Bacillus coagulans* SANK 70258 spores preparation (LACRIS-S), as an ingredient in select food categories, produced consistent with cGMP and meeting appropriate food-grade specifications as presented in the supporting dossier is "Generally Recognized as Safe" (GRAS) based on scientific procedures.

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

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